ANTIBODY FORMATION AND FOLLICULAR IMMUNE COMPLEXES

EXPERIMENTAL AND CLINICAL ANALYSIS *IN SITU*

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ANTILICHAAMVORMING EN FOLLICULAIRE IMMUUNCOMPLEXEN
EXPERIMENTELE EN KLINISCHE ANALYSE *IN SITU*

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Cover: A protective network. The scaffolding for the restoration of the Saint Michael church, Hamburg, FRG, 1990. Final design by Yvonne Witt and Jacky Verheyen.

FEBODRUK ENSCHEDE

... et que la parole humaine est comme un chaudron fêlé, ou nous battons des mélodies à faire danser les ours, quand on voudrait attendrir les étoiles.

... and language is like a cracked kettle we beat out tunes for bears to dance to, while all the time we want to move the stars to pity.

Gustave Flaubert - Madame Bovary (1857)

Aan mijn ouders
Voor Mineke, Harald en Gabri

CONTENTS

ABBREVIATIONS

ADCC	-	antibody-dependent cell-mediated cytotoxicity
AEC	-	3-amino-9-ethylcarbazole
AFC	-	antibody forming cells
AIDS	-	acquired immune deficiency syndrome
AP	-	alkaline phosphatase
ARC	-	AIDS-related complex
β-gal	-	beta-galactosidase
BSA	-	bovine serum albumin
CD	-	cluster of differentation
Cl_2MDP	-	dichloromethylene diphosphonate
CTL	-	cytotoxic T-lymphocytes
EDC	-	1-ethyl-3(3-dimethylaminopropyl)carbodiimide
ELISA	-	enzyme linked immunosorbent assay
FCS	-	fetal calf serum
FDC	-	follicular dendritic cells
gp	-	glycoprotein
HIV-1	-	human immunodeficiency virus type 1
HLA	-	human leukocyte antigen
HRP	-	horseradish peroxidase
HSA	-	human serum albumin
IDC	-	interdigitating cells
IFN-γ	-	interferon gamma
IL	-	interleukin
KLH	-	keyhole limpet hemocyanin
LPS	-	lipopolysaccharide
MBS	-	maleimide-benzoyl-succinimide-ester
MHC	-	major histocompatibility complex
MAb	-	monoclonal antibody
PAb	-	polyclonal antibody
PALS	-	periarteriolar lymphocyte sheath
PBMC	-	peripheral blood mononuclear cells
PBS	-	phosphate buffered saline
PCR	-	polymerase chain reaction
PGL	-	persistent generalized lymphadenopathy
rec	-	recombinant
SP	-	synthetic peptide
TD	-	thymus dependent
TI-1/2	-	thymus independent type 1/2
TNP	-	trinitrophenyl

CHAPTER 1

AIM OF THE STUDY AND INTRODUCTION TO THE EXPERIMENTAL WORK

1.1 ANTIBODIES AND IMMUNE COMPLEXES

Specific protection against disease resulting from infection with pathogens like viruses, bacteriae, fungi and parasites depends on the two arms of the immune system, i.e. antigen-specific antibody producing B-cells and antigen-specific T-cells. The T-cell receptor, which confers antigen-specifity to the T-cell, is a membrane-bound molecule. T-cells carrying the membrane antigen CD4 (cluster of differentiation) are critically involved in the initiation of immune responses by recognition of processed antigen, cytokine production and cell-cell interactions with other lymphoid cell types. CD8-positive cytotoxic T-cells recognize antigenic determinants displayed on foreign, neoplastic or virally infected cells, and mediate killing of these cells or suppression of viral replication. This is called the cellular response.

B-cells produce antibodies, proteins that can recognize and bind molecular structures with high specificity and affinity. This is called the humoral response, as the antibodies are secreted in the body fluids. Antibodies produced by antigen specific B-cells can be regarded as secreted receptors. Antibodies are involved in protection against antigens accessible to the blood and lymph flow. Specificity of the antibody molecule resides in the two identical antigen-binding sites (Fab) located on the two tops of the Y-shaped molecule. Effector functions of antibodies include neutralization of toxins, agglutination of pathogens, virus neutralization, complement activation, opsonization and ADCC (antibody-dependent cell-mediated cytotoxicity). Several of these effector functions are dependent on the tail of the Y-shaped antibody, the Fc-part.

Binding of an antibody to its complementary antigen results in the formation of an immune complex. Circulating immune complexes are rapidly cleared from the body by a number of processes, including ingestion and degradation by mononuclear phagocytes. However, a fraction of these immune complexes localizes in the follicles (germinal centers) of lymphoid organs, where they are involved in antigen presentation for B-cell responses.

Antibodies can also have disease-promoting effects, for example if they (cross-)react with self-antigens, leading to autoimmune phenomena, or when they enhance viral infectivity, through binding and internalization of virus-antibody-complement complexes via Fc- and C3-receptors. Both antibody formation and follicular immune complexes are discussed in detail in chapter 2.

In view of the wide array of functions of antibodies and follicular immune complexes in immunity, a detailed understanding of the mechanisms involved in generation and regulation of antibody formation is necessary. Such understanding will help to manipulate antibody formation to induce protection against infectious and autoimmune diseases. In addition, it will allow improvement of strategies for the

generation of antibodies applicable in research, diagnosis and therapy.

1.2 AIM OF THE STUDY

The aim of the studies presented in this thesis was to investigate the formation of epitope-specific antibody and the mechanisms involved in the localization of resulting antigen-antibody immune complexes in lymphoid follicles. These issues were addressed both in experimental animals and in clinical material. HIV-1 (human immunodeficiency virus type 1) was studied as an infectious pathogen because of its lymphotropic nature, its histopathological effects on the lymphoid microenvironment, and because of the urgent need for HIV-1 specific antibodies for research purposes and a better understanding of the HIV-1 specific antibody response.

1.3 THE *IN SITU* APPROACH

Immunological research relies heavily on *in vitro* methods, a reductionistic approach using isolated and purified cell populations to study lymphoid cell function *in vitro*. However, *in vivo*, different cell types display distinct migration routes and distinct compartments of localization. Cells are exposed to the influence of locally present accessory cells, interleukins and the extracellular matrix. *In vitro* studies necessarily disregard this complex organization of the lymphoid organs. As a consequence, lymphoid cell functions and interactions as seen *in vitro* reflect the full potential of such cells, but the events actually occurring *in vivo* are dictated and restricted by the lymphoid microenvironment. The immunocytochemical *in situ* approach allows the study of *in vivo* events at a given moment in time. Therefore, we chose to address the questions of this thesis with the *in situ* approach, using existing and new (chapter 5) immunocytochemical methods. These methods are explained in figure 1. *In situ* studies offer several distinct advantages: cells are studied in their natural microenvironment, excluding tissue culture artefacts; anatomical localization of antigen, infected cells, accessory cells and antibody forming cells (AFC) can be correlated; results can be quantitated using computer-aided image analysis, and the sensitivity of the techniques used is high in the sense that a single cell can be visualized in a section of a complete organ.

1.4 INTRODUCTION TO THE EXPERIMENTAL WORK

The chapters 4 to 7 describing the experimental work of this thesis are preceded by chapter 2 which provides an overview of the events taking place in the lymphoid microenvironment during the antibody response. Chapter 3 summarizes

those aspects of HIV-1 infection, the HIV-1 specific immune response and vaccine development which are relevant to the studies of this thesis.

Chapter 4 is concerned with the requirements for follicular trapping of immune complexes. In the mouse model, we studied trapping of immune complexes of defined size and composition in splenic follicles. In addition, we studied the possible involvement of splenic macrophages and marginal zone B-cells in trapping of immune complexes.

Chapter 5 describes the development of new methods to study HIV-1 specific antibodies present in antibody forming cells and immune complexes *in situ*. Synthetic peptides were coupled to detector enzymes to allow detection of epitope-specificity of locally present antibody. Different fixation procedures that inactivate HIV-1 were evaluated for application in immunocytochemistry with antibodies and antigen-enzyme conjugates.

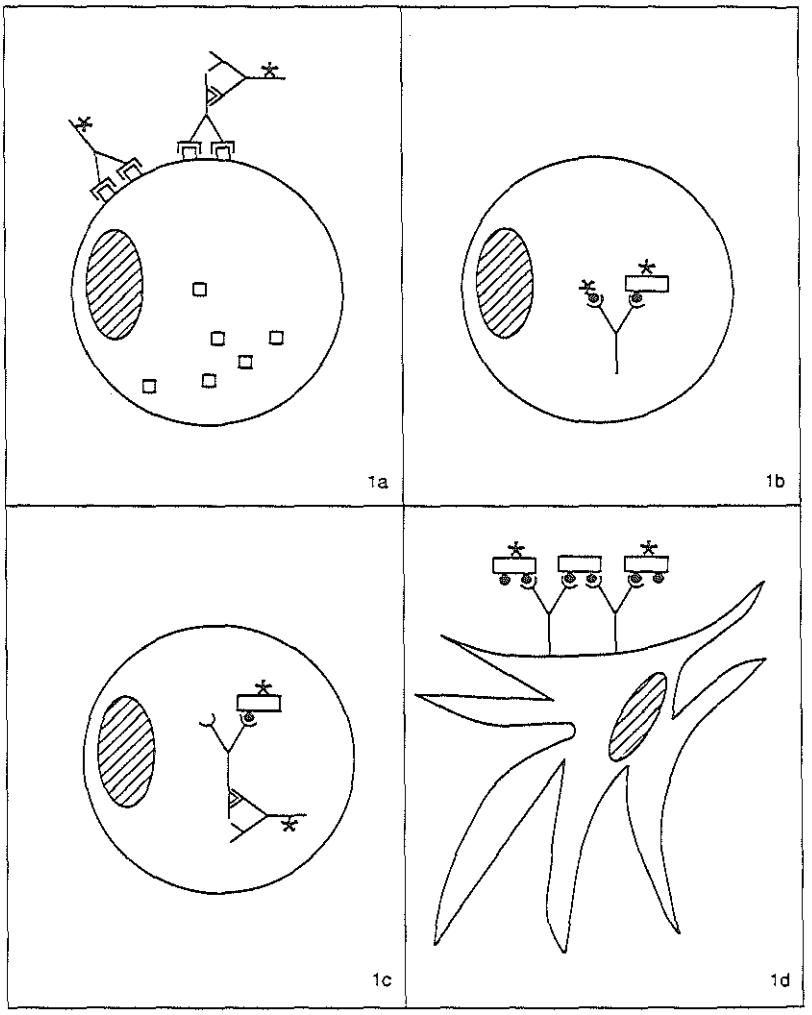
In chapter 6, methods developed in chapter 5 were used to detect HIV-1 specific antibody forming cells in human lymph nodes.

In chapter 7, neutralizing monoclonal and polyclonal antibodies against pre-selected sites within the V3-domain of HIV-1 gp120 were generated with synthetic peptides and extensively characterized in immuno(cyto)chemical and functional assays.

In chapter 8, main points emerging from the experimental studies are discussed in the context of antibody formation, follicular trapping of immune complexes and vaccine development.

FIGURE 1. IMMUNOCYTOCHEMICAL TECHNIQUES USED

1a) Detection of intracellular or membrane associated antigen, using antigen-specific enzyme-labeled antibody as a probe. Detection is either direct using a single antibody, or indirect using a labeled second antibody specific for an isotype determinant. Intracellular antigen can also be detected using this method (not shown for reasons of clarity).
1b) Detection of intracellular or membrane associated antigen-specific antibody, using the corresponding enzyme-labeled antigen as a probe. The antigen in the conjugate can be a protein, a synthetic peptide or a hapten. Protein-specificity, epitope-specificity and hapten-specificity can be determined with these conjugates, respectively.
1c) Simultaneous detection of epitope-specificity and isotype produced. Epitope-specificity is detected as described under b, using a synthetic peptide-enzyme conjugate. The isotype of the antibodies produced by the AFC is determined using an isotype-specific antibody, labeled with another detector enzyme. As the enzymes produce different colours, double staining is possible.
1d) Detection of antigen-specific antibody present in immune complexes in lymphoid follicles. Immune complexes are retained on the surface of follicular dendritic cells (FDC). Using antigen-enzyme conjugates as in Panels b and c, free antigen binding sites can be detected. If all antigen binding sites have already been occupied by antigen *in vivo*, no conjugate will be bound despite the presence of the specific antibody.



cell with nucleus

antibodies of distinct specificities

synthetic peptide or hapten

membrane or cytoplasmic antigenic determinant

isotype-specific determinant

protein antigen

detector enzyme

CHAPTER 2

CELL-CELL INTERACTIONS: IN SITU STUDIES OF SPLENIC HUMORAL IMMUNE RESPONSES

This chapter is adapted from:

CELL-CELL INTERACTIONS: *IN SITU* STUDIES OF SPLENIC HUMORAL IMMUNE RESPONSES

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2.1 INTRODUCTORY REMARKS

AIM OF THE CHAPTER

The aim of this chapter is to concisely discuss the cell-cell interactions occurring in the splenic lymphoid microenvironment leading to the production of antigen-specific antibodies after experimental intravenous immunization. The spleen is an important lymphoid organ, involved in immune reactions against all types of antigens that may appear in the circulation. The central role of the spleen is emphasized by the fact that this organ is quantitatively the main lymphoid organ involved in lymphocyte recirculation (reviewed by Pabst, 1988) and its role in the prevention of opportunistic post-splenectomy infections as demonstrated in autotransplantation studies (cf. Claassen et al., 1989). Its complex anatomical organization with distinct compartments containing specialized cell types, allows for the different cell-cell interactions necessary for the generation of the humoral immune response.

We will focus mainly on the murine spleen, which we have studied in detail using immunocytochemical techniques permitting functional *in situ* studies of structurally intact tissue. It should be noted that inter-species differences in structure and function of lymphoid organs exist (cf. Claassen, 1991b). Extrapolation of animal studies to the human situation should therefore be performed with caution. We will successively discuss the architecture of the spleen, the routing of antigens in the spleen, cell-cell interactions occurring during responses against different types of antigen, the role of antigens trapped in lymphoid follicles, analysis of humoral immune responses in human tissue and the completion of the humoral immune response.

CLASSIFICATION OF ANTIGENS

We will separately discuss the cell-cell interactions required to generate humoral responses against thymus dependent (TD) and thymus independent (TI) antigens because the initial events seem to be different for the responses to these antigens. However, we will argue that the final differentiation of B-cells ultimately leading to plasma cells is identical for these antigens. In this study we will basically divide the antigens in soluble or particulate on the one hand and TD or TI on the other. In table 1, a number of frequently used model antigens is listed with some of their properties.

By definition, B-cells can not respond to TD antigens (proteins, some particulate antigens) without T-cell help. As a consequence, these antigens do not give rise to a response in nude mice because of the lack of a functional thymus. TI

TABLE 1. PROPERTIES OF SOME EXPERIMENTAL ANTIGENS USED IN THE MOUSE

	TYPE OF ANTIGEN	UPTAKE BY	PRESENTATION BY	POLYCLONAL B ACTIVATION	MEMORY	ISOTYPES
THYMUS DEPENDENT						
SOLUBLE	proteins (OVA, BSA, KLH)	rpM	IDC, B-cells	-	+	IgM/IgG
	and peptides (> ± 15 AA)			-	+	"
PARTICULATE	liposomes with protein	mzM (rpM ?)	?	-	+	"
	SRBC	rp	IDC, B-cells	-	+	"
	Lactobacillus	mzM	FDC,IDC,fB-cells	-	+	"
THYMUS INDEPENDENT TYPE 1						
SOLUBLE	LPS	rpM	B-cells direct	+	±	IgM
	Dextran-sulfate	?	B-cells direct	+	-	IgM
PARTICULATE	Brucella abortus	mzM	mz and fB-cells	+	±	IgM/IgG
THYMUS INDEPENDENT TYPE 2						
SOLUBLE	dextran	mzM	FDC, fB-cells	-	-	IgG3/IgG1
	Ficoll (and HES)	mzM	FDC, fB-cells	-	±	"
	detoxified LPS	rpM	?	-	±	"
PARTICULATE	liposomes (no protein)	mzM	mzB-cells, FDC	-	-	none
	haptenated liposomes	mzM	mzB-cells, FDC	-	-	IgM

± = still under discussion, IDC = interdigitating cells, rp = red pulp, mz = marginal zone, M = macrophage, rpM = red pulp macrophages, mzM = marginal zone macrophages, FDC = follicular dendritic cells, fB-cells = follicular B-cells, BSA = bovine serum albumin, OVA = ovalbumin, KLH = keyhole limpet hemocyanin, SRBC = sheep red blood cells, BCG = Bacille Calmette Guerin, LPS = lipopolysaccharide, HES = hydroxyethyl starch. Gerritse et al. (1990, 1991) have described the use of Lactobacillus as antigenic carrier. Some of the listed interactions of antigens with different cell types are still speculative.

antigens on the other hands can directly stimulate B-cells to proliferate and differentiate into plasma cells, by definition without requiring T-cell help. Direct activation can be achieved either by a mitogenic component (e.g. lipid-A in lipopolysaccharide [LPS]), or by crosslinking of antigen specific membrane immunoglobulins on B-cells. This is due to the repetitive structural character of the antigen, as in the polysaccharide of bacterial capsules. The different modes of direct activation are reflected in the subdivision of TI antigens in types 1 and 2. The bacterial capsule consisting of polysaccharide and lipid-A is the typical TI-1 antigen. TI-2 antigens are model antigens which do not occur in nature: they are obtained by detoxifying TI-1 antigens by removing the lipid-A component. The polysaccharide Ficoll is the most widely used TI-2 antigen. Both TI-1 and 2 antigens elicit a response in nude mice, but TI-2 antigens do not elicit a response in CBA/N mice (Scher, 1982). Responses to TI-2 antigens require an intact spleen but *in vitro* studies by several groups did not provide satisfactory data for a consensus on whether a single cell type was responsible for this splenic dependence (cf. Claassen et al., 1989; 1991a).

The seemingly contradictory results on the involvement of T-cells and T-cell factors led to the proposals to classify TI-1 and TI-2 antigens depending on their *in vivo* behaviour and relation to marginal zone cell types (Claassen et al., 1987) or their requirement for lymphokines (TI-2) or lack thereof (TI-1) (Goud et al., 1988), rather than the initial classification based on immunological response in certain mouse strains (Chused et al., 1976). In this paper, however, we will adhere to the classical subtyping as described by Chused et al.

2.2 ARCHITECTURE OF THE SPLEEN

Macroscopically, the spleen can be divided into the red and the white pulp. The red pulp consists of erythrocytes and nucleated cells like lymphocytes, megakaryocytes and macrophages (Van Rooijen et al., 1989b). The white pulp contains immunocompetent cells in which three compartments can be distinguished: the periarteriolar lymphocyte sheaths (PALS), the follicles and the marginal zone. The structure of the spleen is directly related to the vascularization of this lymphoid organ (figure 2): the spleen has an open circulation and a closed circulation. In the open circulation, blood enters the spleen at the hilus through the splenic artery, which ramifies into trabecular arteries. Small arterial vessels leave the trabeculae as so-called central arterioles, which become gradually surrounded by lymphatic tissue, called the periarteriolar lymphocyte sheath (PALS).

The follicles are globular structures attached to the PALS and consist mainly of resting B-cells and follicular dendritic cells (FDC). The marginal zone, which surrounds the PALS and the follicles, contains B-cells, macrophages and relatively

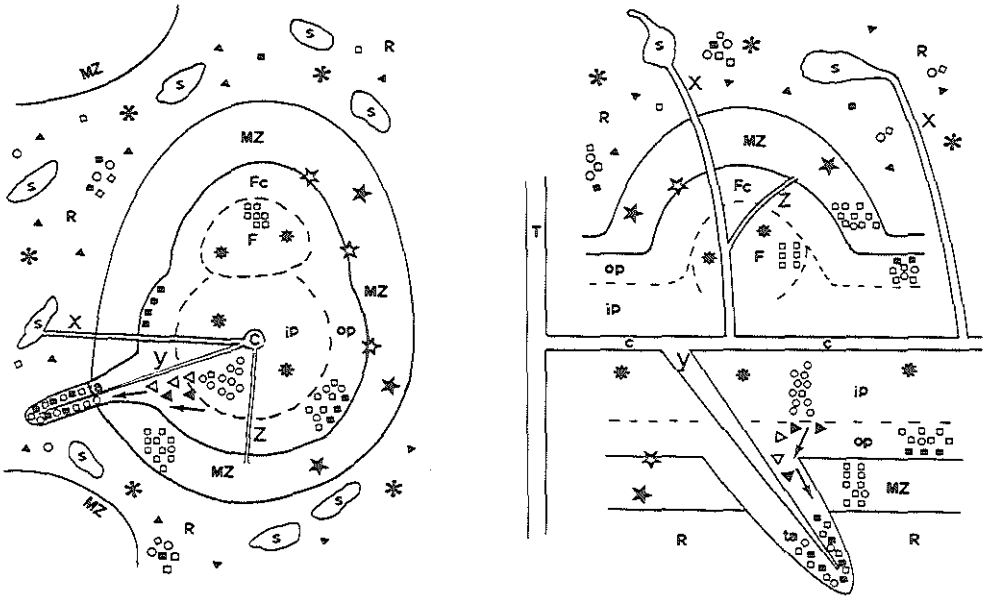
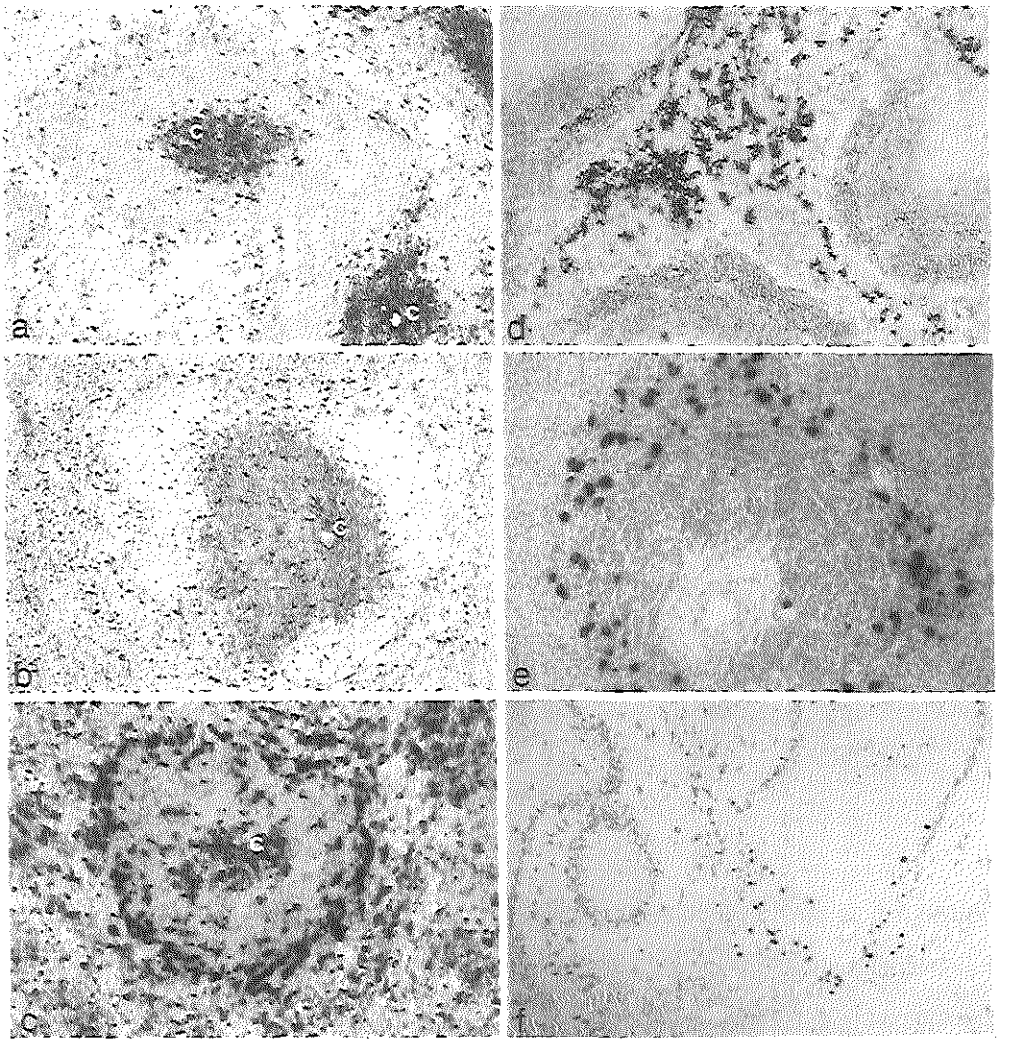


FIGURE 2. SCHEMATIC REPRESENTATION OF THE ORGANIZATION OF THE MURINE SPLEEN

C = central arteriole, ip = inner PALS, op = outer PALS, F = follicle or (germinal) center, Fc = follicle corona, MZ = marginal zone, T = trabecular artery, R = red pulp, S = sinus, ta = lymphoid sheath around terminal arteriole. Open squares = B-cells, closed squares = antibody forming cells (AFC), open triangles (in op) = migrating B-lymphoblasts, circles = T-cells, closed triangles (in op) = migrating T-blasts, closed stars (in MZ) = marginal zone macrophages, open stars = marginal metallophilic macrophages, double stars (in F) = follicular dendritic cells (FDC), square double stars (in ip) = interdigitating cells, asterisks (in rp) = megakaryocytes, triangles (in rp) = acid phosphatase positive red pulp macrophages.
Reticular fibroblasts and transient blood cells in the red pulp, such as erythrocytes, thrombocytes, granulocytes and monocytes, are not shown.
X represents a bloodvessel shunting the blood directly into a sinus, Y represents a terminal arteriole opening into the red pulp, Z represents a terminal arteriole opening into the marginal zone.
The B and T lymphoblasts as indicated by open and closed triangles, respectively, are in a stage of development where they can not yet be detected using antigen-enzyme conjugates and cytokine specific antibodies. Arrows indicate the direction of migration of these lymphoblasts.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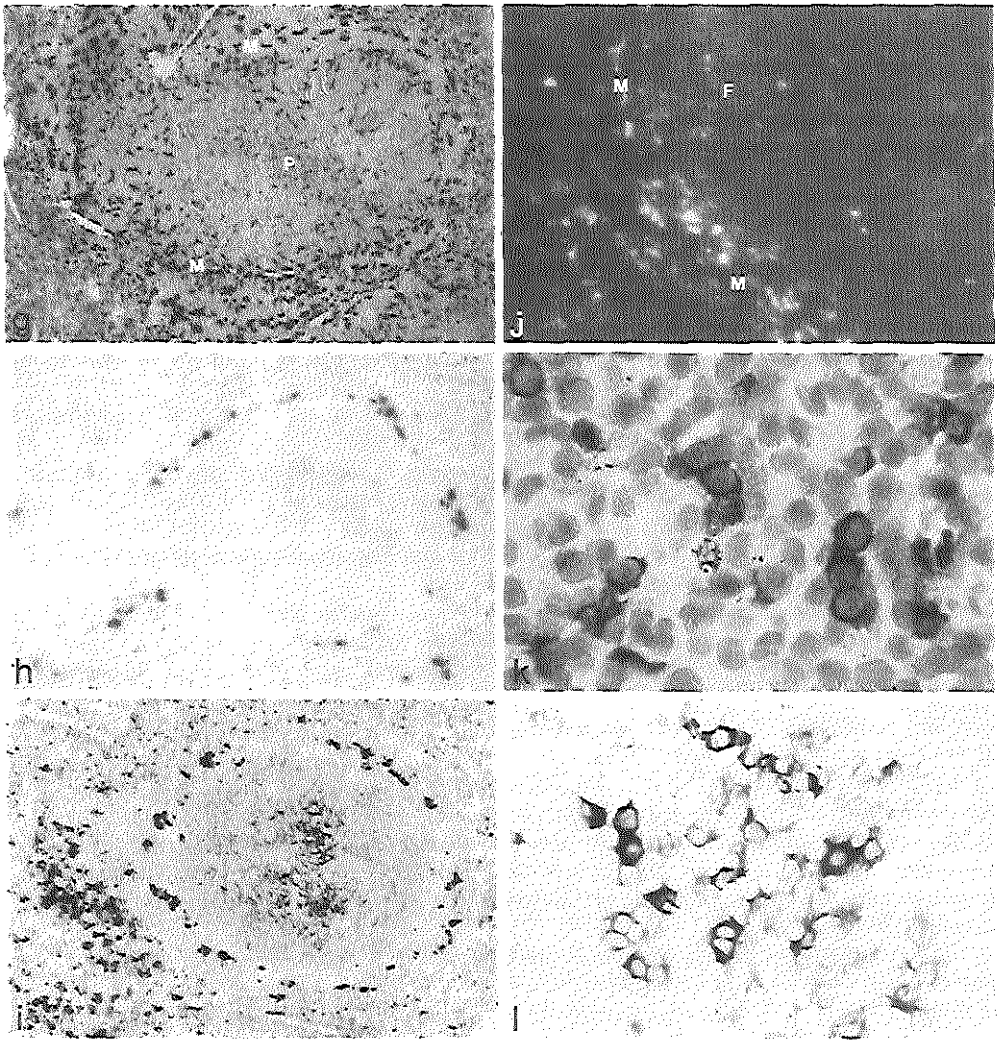
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FIGURE 3. IMMUNOCYTOCHEMISTRY OF SPLENIC CRYOSTAT SECTIONS

8 μm sections of murine spleen are shown (unless indicated otherwise) after acetone fixation and incubation with monoclonal antibodies and/or antigen-enzyme conjugates. C = central arteriole, f = follicle, m = marginal zone, p = periarteriolar lymphocyte sheath (PALS), r = red pulp, t = terminal arteriole. Unless otherwise indicated, immunocytochemical techniques have been described in detail by Van Rooijen et al. (1989b). All photomicrographs have been taken with an Olympus Vanox-S/AH-2 photomicroscope.

3a) Brown: T-cells in the inner PALS (Thy-1/HRP; Thy-1 has been described by Ledbetter and Herzenberg, 1979). Blue: B-cells in the outer PALS (anti-mouse-IgM/HRP; anti-IgM has been described by Braun and Unanue, 1980) Note: few T-cells are present in the MZ and almost none in the follicles.
3b) Brown: T-cells (as in Fig. 3a). Red: all splenic macrophages demonstrated by staining for endogenous acid phosphatase activity.
3c) Dark ring: T-cells (as in Figs. 3a and 3b). Red spots: marginal metallophilic macrophages stained by revelation of endogenous non-specific esterase activity.
3d) Red: B-cells, showing both membrane and cytoplasmic staining (anti-IgM/HRP; Braun and Unanue, 1980). Blue/violet: anti-TNP AFC 5 days after immunization with TNP-KLH (TNP-AP).
3e) Rat spleen. Blue: anti-TNP AFC 5 days after immunization with TNP-KLH (TNP-AP).
3f) Rat spleen. Blue: anti-TNP AFC; red: marginal zone and metallophilic macrophages (ED3/HRP) 5 days after immunization with TNP-Ficoll.
3g) Red: all splenic macrophages after staining for endogenous acid phosphatase. Black: all phagocytic cells in black by functional uptake of intravenously administered carbon particles.
3h) Follicular localization of TNP-Ficoll. Blue-green marginal zone macrophages (cytoplasmic staining) and follicularly trapped antigen (anti-TNP/β-gal) 5 days after immunization with TNP-Ficoll. Note also small spots of antigen in red pulp macrophages.
3i) Follicular localization of TNP-Ficoll. Brown: antigen (anti-TNP/HRP) Red: anti-TNP AFC and follicular immune complexes (TNP-AP), and violet: interferon-γ producing cells (DB-1/AP; refer to Van den Eertwegh et al., 1991b, for experimental detail).
3j) Section made 24 hours after intravenous administration of liposomes labeled with a yellow fluorescent hydrophobic label (DiI). Yellow: marginal zone macrophages, displaying intense cytoplasmic labeling after uptake of liposomes, and surface binding of liposomes to follicular B-cells (Claassen, in press, a).
3k) Human lymph node biopsy of an HIV-1 infected individual. Red: p24-specific AFC (p24-HRP; refer to Laman et al., 1991b, for experimental detail).
3l) Detail of Fig. 3i. Note direct juxtaposition of specific anti-TNP AFC (blue) and IFN-γ producing cells (red), suggesting a direct involvement of IFN-γ in the regulation of antibody production.

few T-cells.

Small terminal arterioles, also called marginal zone bridging channels (indicated as Y in figure 2), traverse the white pulp (PALS and marginal zone) and open into the red pulp, where they are surrounded by small sheaths of active lymphoid cells. In the red pulp, the blood flows in small channels, formed by reticular cells, and is subsequently collected by the venous sinuses which drain into the efferent splenic venule. Other terminal arterioles (indicated as Z in figure 2) open directly into the marginal zone, where due to the reduced flow the first cell-cell interactions between immunocompetent cells like T-cells, B-cells, and macrophages may occur.

A third type of terminal arterioles (indicated as X in figure 2) shunts the arterial blood flow directly through arterioles into the efferent venule. This latter type forms the closed circulation of the spleen.

In the mouse, the PALS can be further subdivided into the inner PALS and outer PALS. The inner PALS consists mainly of resting T-cells mainly (figure 3a). There is no preferential localization of T-helper (CD4+) or T suppressor-cytotoxic (CD8+) cells (Claassen et al., 1989), but no CD8+ cells are present in the B-cell domains. Furthermore, interdigitating cells (IDC) are also found in the inner PALS. These are shown in figure 3b as weakly acid phosphatase positive red spots in the T-cell area around the central arteriole. IDC are strongly MHC (major histocompatibility complex) class II (Ia) positive. Studies with two different monoclonal antibodies against dendritic cells have shown that IDC *in situ* are identical to the dendritic cells isolated *in vitro* (Breel et al., 1987). Dendritic cells *in vitro* are extremely potent accessory cells in the induction of T-cell dependent responses (Inaba and Steinman, 1987); this potency therefore seems to compensate for their low frequency of occurrence.

The outer PALS is predominantly populated by B-cells and some T-cells (figures 3a-c) and macrophages (figure 3b). A special subset of macrophages can be found at the border of the PALS and the marginal zone. In view of their staining with silver they were termed marginal metallophilic macrophages. These cells are actually located in the marginal zone with processes protruding into the outer PALS. They contain non-specific esterase (figure 3c; Eikelenboom et al., 1978) and can be stained for acid phosphatase (figure 3b), but they have low phagocytic capacity (figures 3g and j). In the mouse they are specifically recognized by the monoclonal antibody MOMA-1 (Kraal et al., 1986). Antibody forming cells (AFC) in the spleen are found mainly around the terminal arterioles (figure 3d), but also in high numbers in the outer PALS (figures 3d-e).

Positioned in a concentric ring around the marginal metallophilic macrophages, another macrophage subset can be found within the marginal zone: the marginal zone macrophages. These macrophages are Ia negative, acid

phosphatase positive and have extraordinary phagocytic capacity combined with a strategic localization with respect to the white pulp cappillaries, allowing them to meet and ingest antigens efficiently. In the mouse they are recognized by monoclonal antibody ERTR-9 and in the rat by ED3 (which also recognizes marginal metallophilic macrophages, figure 3f). These cells can also be recognized by their uptake of TI-2 antigens such as the soluble antigen TNP-Ficoll (trinitrophenyl-Ficoll)(figures 3h and i), or particulate antigens like haptenated liposomes (figure 3j). The marginal zone further consists of a particular population of B-cells which are μ-positive and δ-negative (figure 3a; MacLennan et al., 1982).

Follicles mainly consist of resting B-cells, easily identified with an anti-IgM antibody as shown in figures 3a and d. Primary follicles show an even staining pattern and predominantly consist of small B-cells (figure 3a). Upon antigenic stimulation one observes the generation of secondary follicles, also called 'germinal centers', consisting of a follicle center with resting cells and a follicle corona (or mantle zone) with blast-like activated B-cells (figure 3d). The follicles are interspersed with follicular dendritic cells (FDC), a cell type specifically recognized by monoclonals like ED5 in the rat (Jeurissen and Dijkstra, 1986) or DRC-1 in man. The dendrites of the FDC form an intricate network, called the follicular web, where follicular B-cells lie in close assocation with the FDC. This close contact hampers immunocytochemical studies of, for example, expression of cell surface markers by FDC, because it is difficult to establish to which cell the stained membranes actually belong. In addition, the close association of follicular cells complicates the purification of intact FDC for *in vitro* studies (cf. Heinen et al., 1988). FDC are thought to present antigens in the form of immune complexes, which they retain on their surface by Fc and C3 receptors. Localization of antigens/immune complexes on FDC can be observed during natural infections (e.g. Rácz et al., 1989) and experimental immune responses (figure 3i). The trapping ability of FDC can also be used to functionally visualize this cell type by administering *in vitro* preformed immune complexes, such as peroxidase-anti-peroxidase (PAP)(cf. Laman et al., 1990a). Mechanism and function of follicular trapping of antigens are discussed in detail below.

2.3 ANTIGEN ROUTING AND HANDLING IN THE SPLEEN

ENTRANCE OF ANTIGENS INTO THE SPLEEN

Antigens reaching the spleen via the circulation may have entered the body directly into the bloodvessels. Antigens may also reach the spleen indirectly, i.e. they may gain access to the lymph flow anywhere in the body and subsequently reach the circulation e.g. via the ductus thoracicus after passing one or more lymph nodes. Such antigens are carried into the spleen by the splenic arteries and are discharged

from the terminals of the white pulp capillary network, between the cells in the marginal zone. This marginal zone separates the white pulp from the red pulp and plays an important role in the distribution of cells, antigens and immune complexes over the different compartments of the spleen.

An alternative pathway for the introduction of antigens into the spleen is provided by the IDC of non-lymphoid organs and the Langerhans cells of the skin. Particulate antigens can be processed by resident macrophages in non-lymphoid tissues and transferred to IDC, which transport the antigen (Austyn et al., 1988a; Larsen et al., 1990) and present it to T-cells in the PALS of the spleen. Analogously, Langerhans cells in the skin pick up antigens directly or after processing by skin macrophages. Subsequently they migrate through the lymph vessels, where they are called veiled cells, to eventually present the antigen in the lymph nodes and spleen, where they are called IDC.

HANDLING OF ANTIGENS BY SPLENIC LYMPHOID CELLS

Antigens that have arrived in the splenic marginal zone will be ingested by macrophages in the marginal zone itself or, after following the main blood flow to the red pulp, by the red pulp macrophages. A small proportion of the antigens is carried into the white pulp. This part may be ingested by the marginal metallophilic macrophages at the outer border of the white pulp or by the white pulp macrophages in PALS and follicles. The main fraction of such macrophage-ingested antigens is completely degraded, but a small proportion of the ingested antigens is processed and presented to the T-cells of the immune system (Unanue, 1984). Other antigen presenting cells are the interdigitating cells (IDC) in the central parts of the PALS and the B-cells.

Both of the latter cell types are much more efficient in processing and presentation of small soluble antigens than macrophages, but they seem not to be able to process large particulate antigens. For example, protein antigens encapsulated in liposomes cannot be processed by B-cells owing to their inability to internalize and degrade the encapsulated antigen (Dal Monte and Szoka, 1989). Just like B-cells, IDC cannot process those antigens that require extensive intracellular degradation. Processing of particulate antigens by macrophages, followed by transfer of the processed antigens to IDC or B-cells, which in turn present the (possibly further processed) antigens to T-cells, is a well-established phenomenon *in vitro* (Roska and Lipsky, 1985; Kapsenberg et al., 1986; Wright et al., 1987). However, as yet there is little evidence that this transfer also occurs *in vivo*. Macrophages are required for the immune response against liposome-associated antigens (Claassen et al., 1987; Su and Van Rooijen, 1989). An obligatory role for macrophages has also been demonstrated in, for example, the immune

response against particulate bacterial antigens (Ziegler et al., 1987).

Probably because of their strategic position at the end of the white pulp capillaries (Van Rooijen et al., 1989b), marginal zone macrophages ingest a large proportion of all particles entering the spleen. Recent observations support the idea that marginal zone macrophages are required for the processing of the particulate TD antigen trinitrophenylated sheep red blood cells (TNP-SRBC) (Delemarre, 1990). B-cells lie between the cytoplasmic processes of marginal zone macrophages (Dijkstra et al., 1985), a location which may facilitate the transfer of processed antigens to the latter cells. The strong adherence of B-cells to freshly isolated marginal zone macrophages has been demonstrated *in vitro* (Humphrey and Grennan, 1981), confirming the interaction between these cell types. For IDC which are already present in the spleen, the most obvious mechanism is that the IDC-T-cell clusters in the inner PALS obtain the processed particulate antigens from white pulp macrophages that are present in the inner PALS (Van Rooijen et al., 1989b).

Recently, an alternative route of antigen presentation to B-cells was described (Szakal et al., 1989), by means of immune complex coated beads (iccosomes) which detach from the dendrites of FDC.

Using TNP-specific antibodies, we found that TNP-Ficoll, a TI-2 model antigen, is located in the marginal zone macrophages as well as in the follicular areas shortly after intravenous injection. The marginal zone macrophages take up TNP-Ficoll and retain it for long periods of time (up to several weeks). The follicular localization of TNP-Ficoll is strong shortly after injection but starts to decrease gradually after a few days (Van den Eertwegh et al., submitted). The follicular localization of TNP-Ficoll may represent binding to follicular dendritic cells and/or follicular B-cells and may be the result of complement activation by the antigen (see also part V).

In conclusion, the fate of an antigen arriving in the spleen is largely dependent on its characteristics, i.e. soluble vs. particulate, protein vs. polysaccharide, complement-activating capability, complex-formation with antibody.

2.4 CELL-CELL INTERACTIONS DURING THYMUS-DEPENDENT ANTIBODY RESPONSES

CELL TYPES REQUIRED FOR HUMORAL RESPONSES TO TD ANTIGENS

Initial studies on the localization of the humoral immune response in the spleen were based on staining of reactive (blastlike) lymphoid cells with methylgreen and pyronin for DNA and RNA, respectively, (Langevoort, 1963) and demonstration of Ig containing cells (Van Ewijk et al, 1977; Eikelenboom et al., 1982). These studies already pointed to a development of Ig-producing cells (of the B-cell lineage) in the peripheral parts of the PALS, and the development of lymphoblasts (of the T-cell

lineage) in the central parts of the PALS. Lymphoid follicles, comprising the remaining white pulp, also showed a clear histological blast cell reaction upon antigen administration (Langevoort, 1963). Their germinal centers, characterized by the large scale proliferation of B-cells, were thought to be involved in the generation of B-memory cells (reviewed by Thorbecke, 1990).

The marginal zone, PALS and follicles are compartments where cell-cell interactions occur and in these areas the humoral immune response will be initiated. It is now generally agreed that at least three different cell types are involved in antibody responses against thymus dependent antigens. Cells of the B-lineage are ultimately producing the specific antibodies. These B-cells are 'helped' by T-cells (the CD4+ T-cells). The CD4+ T-helper cells in turn require the processing and the presentation of the fragmented TD antigens by accessory cells, such as macrophages, IDC or B-cells, depending on the nature of the antigen. Much effort has been devoted to determine the relative contribution of different accessory cells to immune responses against distinct antigens.

A role for macrophages in the processing of antigens as an initial step in immunity has been the subject of numerous studies since the early 1960s (Weissman and Dukor, 1970; Unanue, 1984; Delovitch, 1988; Katz, 1988). In the 1970s IDC were identified and were shown to be able to present antigens to T-cells (Steinman and Nussenzweig, 1980). The last decade has seen a rapidly increasing body of literature on B-cells as being higly efficient in processing and presentation of antigens to T-cells, through the use of their membrane-associated antigen-specific immunoglobulins (Ashwell, 1988; Abbas, 1988; Manca et al., 1988). We have shown that soluble protein antigens like human serum albumin (HSA), which failed to elicit a detectable antibody response when injected intravenously in mice, did induce a substantial response when they were targeted to macrophages by incorporation in liposomes. *In vivo* elimination of the macrophages in the spleen reduced the response significantly (Claassen et al., 1987; Su and Van Rooijen, 1989). From these studies it appears that B-cells and dendritic cells cannot handle certain (particulate TD) antigens without pre-processing by macrophages. In conclusion, the characteristics of the antigen determine which cell type will function as the principal accessory cell, viz. particulate antigens by macrophages and soluble antigens by IDC and B-cells.

INITIATION OF THE HUMORAL RESPONSE TO TD ANTIGENS

Expression of surface markers, cell-cell contact and cytokine secretion by accessory cells and T-cells are essential requirements for the differentiation process of B-cells, as demonstrated by *in vitro* studies (Noelle and Snow, 1990; Parker, 1990). These studies showed that direct physical cell-cell contact between antigen

presenting B-cells, macrophages or IDC, and T helper cells is required for activation of T-cells. Activation induces the expression of novel surface antigens on these T-cells, which provide the major growth stimulus to B-cells. This event induces enhanced B-cell RNA synthesis and the development of B-cell responsiveness to cytokines, which are produced by activated T-cells. These cytokines act at distinct steps in the B-cell activation pathway (effects of cytokines on T-cells are not discussed here). Cytokines like IL-1, IL-2 and IL-4 support the growth and proliferation of activated B-cells, while others, such as IL-4, IL-5, IL-6 and IFN-γ act as differentiation factors (Abbas, 1988). Kupfer et al. (1991) recently showed that after *in vitro* mixing of antigen specific T-helper cells and B-cells, T-helper-B-cell couples were formed. This interaction resulted in a local, polarized cytokine production by these T-cells at the contact-site with the antigen-specific B-cells. They proposed that T-cells are able to induce selective and specific B-cell responses in this way. *In vivo* studies with cytokines and cytokine-neutralizing monoclonal antibody have already provided evidence on the role of cytokines in immunoglobulin isotype selection (Finkelman et al., 1990).

Recently, we demonstrated that during the humoral response against the TD antigen TNP-Bacille Calmette Guerin (TNP-BCG), T-cells producing IFN-γ are localized in the outer PALS, around the terminal arterioles, and in the red pulp (Van den Eertwegh, 1991a). These data are supported by the findings of Gessner et al. (1990), who found that IFN-γ producing cells are localized in small clusters predominantly in the white pulp and less frequently in the red pulp of spleens of mice infected with lymphocytic choriomeningitis virus, which evokes a TD antibody response. This is in accordance with data of Claassen et al. (1986e), who demonstrated that the majority of specific antibody forming cells directed against a variety of TD and TI antigens were also localized in these compartments, suggesting that T-cell-B-cell interactions may take place in the outer PALS, terminal arterioles and red pulp.

ANTIBODY PRODUCTION BY TD ANTIGEN SPECIFIC B-CELLS

As discussed above soluble antigens may be processed directly by marginal zone B-cells, while macrophages located in the marginal zone of the spleen ingest particulate antigens and transfer processed antigen to neighbouring B-cells. B-cells in general reach the spleen by the circulation, locate in the marginal zone and subsequently reach the PALS and the follicle (Van Ewijk and Nieuwenhuis, 1985). It has been suggested that antigen-specific B-cells, upon antigenic stimulation, migrate from the marginal zone into the adjacent outer parts of the PALS (Van Rooijen et al., 1986b; Liu et al., 1988). On arrival in the PALS, B-cells encounter numerous T-cells, among which are T-cells of the appropriate antigen-specificity. The chance of

antigen-specific B and T-cells meeting is optimized by this migration (cf. Van Rooijen et al., 1986b; Van Rooijen, 1990a). Close T-cell-B cell contact may be required for effective signalling by cytokines. As a result of T-cell help, antigen-specific B-cells differentiate into specific antibody forming cells (AFC).

The fact that a large proportion of all B-cells in the outer PALS ultimately differentiate into AFC implies that these antigen-reactive B-cells were preselected before entering the PALS. Antigen-specific B-cells are thus greatly enriched in the small population of B-cells in the outer PALS. In addition, the ratio between specific T-cells and non-specific T-cells in the adjacent inner PALS is also enhanced because of the antigenic stimulation of T-cells occurring in the IDC-T-cell clusters. One of the most restrictive factors in the inductive mechanism of antibody production - the chance that the antigen-specific B-cell meets a T-cell of appropriate specificity - is thus postulated to be promoted and regulated by the microenvironment in a very efficient way.

Using antigen-enzyme conjugates for detection, AFC are generally first detected in the outer PALS. This is irrespective of the type of antigen used, the primary or secondary character of the response, or the isotype produced.
How do the IDC, with their characteristic localization between the T-cells in the inner PALS (Veerman and Van Ewijk, 1975), fit into this scheme? Rapid clustering of T-cells and IDC has been shown to occur *in vitro* independently of antigen and MHC (Inaba and Steinman, 1986). It is assumed that upon contact with antigen, non-antigen specific cells leave the clusters while antigen-specific T-cells are activated (Austyn et al., 1988b). Given the strategic position of the T-cells (in contact with the IDC in the inner PALS and the antigen-presenting B-cells in the outer PALS), it is possible that both T-cell-B-cell and IDC-T-cell contact are required for optimum induction of an antibody response. If so, the most obvious sequence of events is that the IDC-T-cell contact in the inner PALS takes place before the T-cell-B-cell contact in the outer PALS, because the latter seems to represent the last cell interaction of the B-cells before their differentiation into antibody-forming cells. Alternatively, specific B-cells may contact the T-cells while these are still in the IDC-T-cell clusters.

The IDC-T-cell and the B-cell-T-cell interactions may well have different effects on T-cells. The induction of proliferation of antigen-specific T-cells may be the main function of the IDC-T-cell clusters (Van Ewijk et al., 1977). Since the ratio of antigen-specific over non-antigen-specific T-cells increases as a result of this proliferation, the chance that antigen-presenting B-cells may meet the appropriate T-cells is enhanced accordingly. The physical contact of the T-cell with the antigen-specific B-cell may induce the actual release of cytokines that regulate antibody production by the B-cell.

2.5 CELL-CELL INTERACTIONS DURING THYMUS-INDEPENDENT ANTIBODY RESPONSES

CELL TYPES REQUIRED FOR THE HUMORAL RESPONSE AGAINST TI-ANTIGENS

The spleen plays an important role in the protection against bacterial pathogens such as *Streptococcus pneumoniae*, *Neisseria meningitides* and Haemophilus influenzae (Amlot and Hayes, 1985). Following splenectomy, patients are at high risk for fulminant infections due to these bacteria. Specific antibodies providing protective immunity against these bacterial infections are mainly directed against the polysaccharide component of the bacterial capsule. The polysaccharide component is classified as a TI-2 antigen (Rijkers and Mosier, 1985). The polysaccharide with the lipid-A is a TI-1 antigen. The presence of the spleen seems to be important in the primary encounter of this antigen, whereas secondary responses can also take place at sites outside the spleen (Koch et al., 1982).

The involvement of the spleen in TI-2 responses has led to the suggestion that specific subsets of B-cells are present in the spleen or that another component of the splenic microenvironment is crucial for the response of B-cells to TI-2 antigens. In particular the marginal zone macrophages of the spleen have received much interest in this respect. These macrophages, which specifically take up and retain carbohydrate macromolecules such as TNP-Ficoll (a TI-2 model antigen), have been suggested to play a role in the processing and presentation of TI-2 antigens (Humphrey, 1985). However, macrophage elimination studies (Claassen et al., 1986c 1987; 1988b), studies using splenic autotransplants (Claassen et al., 1989) and functional blocking of polysaccharide uptake by marginal zone macrophages (Kraal et al., 1989) showed that the presence and polysaccharide uptake by these cells are not required for TI-2 responses. Functional data on the function of marginal metallophilic macrophages are scarce, but neonatal injections with antibody MOMA-1, which specifically recognizes these macrophages, suppressed the antibody response to TD and TI-1, but not to TI-2 antigens (Kraal et al., 1988).

INITIATION OF THE HUMORAL RESPONSE TO TI ANTIGENS

As mentioned above, we recently demonstrated that shortly after injection of TNP-Ficoll, the antigen is located in the marginal zone macrophages as well as in the follicular areas. The marginal zone macrophages take up TNP-Ficoll and retain it for long periods of time (up to several weeks). The follicular localization of TNP-Ficoll is strong shortly after injection but starts to decrease gradually after a few days (Van den Eertwegh et al., submitted). The follicular localization of TNP-Ficoll may represent binding to follicular dendritic cells and/or to follicular B-cells. At the

lightmicroscopical level we cannot discriminate between these two cell types. This could implicate that TNP-Ficoll may be able to activate B-cells directly, without requiring a processing/presentation step by marginal zone macrophages. This is in agreement with previous experiments in which marginal zone macrophages were eliminated *in vivo* without affecting the TI-2 immune response (Claassen et al., 1987; 1988b; Kraal et al., 1989). Elimination of macrophages resulted only in an increase of the humoral immune response when lower doses of TNP-Ficoll were used. This indicates that marginal zone macrophages ingest the antigen so effectively that they limit the amount of antigen needed for a maximal response (Claassen et al., 1991a).

A specific role of B-cells in the TI-2 immune response was already suggested by experiments with neonatal mice and CBA/N mice, which carry an X-linked immunodeficiency. TNP-Ficoll was not able to activate B-cells of these mice, which are also characterized by the absence of the Lyb-5+ antigen (Scher, 1982). Letvin et al. (1981), Morrisey et al. (1981) and Fultz et al. (1989) have shown that Ia-expressing cells are required for the TI-2 response. Marginal zone macrophages do not express this molecule, whereas marginal zone B-cells do.

On the basis of data discussed above, we hypothesize that the following events initiate the humoral immune response to TNP-Ficoll after intravenous administration: 1. TNP-Ficoll binds quickly to marginal zone macrophages, FDC and/or follicular B-cells. 2. B-cells may be either stimulated directly, or the FDC present TNP-Ficoll to antigen-specific B-cells, which subsequently bind TNP-Ficoll. 3. TNP-Ficoll drives these resting B-cells into the G_1 stage of the cell cycle, resulting in responsiveness to T-cell derived lymphokines (Monroe et al., 1983). The uptake of TNP-Ficoll by marginal zone macrophages is analogous to the uptake, killing and processing of capsular bacteriae during infections. The follicular localization of TNP-Ficoll may represents the binding of capsular bacteriae and/or capsular antigens from bacteriae, released by the marginal zone macrophages, to FDC and/or B-cells.

The role of marginal zone macrophages in the response against TI-2 antigens is thus not primarily antigen-presentation, but rather removal and degradation of antigen/bacteria, reducing the total antigen load and also the amount of antigen available for presentation. The actual initiation of the humoral response against TI-2 antigens is most likely a function of FDC and B-cells.

ANTIBODY PRODUCTION BY TI ANTIGEN SPECIFIC B-CELLS

Primed B-cells migrate to the outer PALS and follicles of the spleen. These primed B-cells can have cell-cell contact with T-cells of appropriate specificity located in the inner PALS, which are subsequently activated and secrete cytokines. In turn, these cytokines stimulate the G_1 B-cells to proliferate and differentiate into specific antibody forming cells. These differentiating specific B-cells and activated T-cells

leave the white pulp along the sheaths of lymphoid tissue surrounding the terminal arterioles (figure 2). Some of these specific differentiating cells stay in the lymphoid tissue surrounding the terminal arterioles and develop into specific antibody forming cells, releasing specific antibody into the bloodstream. Another part of the specific AFC leave the spleen through the red pulp to the circulation and migrate into extrasplenic sites, such as the bone marrow and other lymphoid tissues. The activated T-cells differentiate and exert regulating and differentiating effects on the antigen-specific B-/antibody-forming cells partly by direct cell-cell interaction and/or by release of cytokines during physical interaction with B-cells (Van den Eertwegh and Claassen, 1991c).

We recently investigated whether IFN-γ is involved in TI-2 responses as *in vitro* data indicated a downregulating role of this cytokine (cf. Van den Eertwegh et al., 1991b). By means of immunohistochemical techniques we found that IFN-γ is active in TI-2 immune response and is produced predominantly by T-cells (either CD4+ or CD8+) and to a lesser degree by natural killer cells. During the peak of the immune response antigen-specific antibody forming cells were juxtaposed to T-cells producing IFN-γ in the outer PALS, terminal arterioles and red pulp (figures 3i and 3l), suggesting a direct cell-cell interaction. DeKruyff et al. (1985) showed that a direct interaction between antigen-specific T-cells with antigen-activated B-cells is a prerequisite for T-cell activation and cytokine production by T-cells in TI-2 immune responses *in vitro*. Our experiments suggest that antibody forming cells may be regulated by IFN-γ, produced by T-cells, and possibly by direct cell-cell interaction with T-cells. The late appearance of IFN-γ producing cells and the juxtaposition of these cells to TNP-AFC is more suggestive for a role of IFN-γ in regulation than for involvement in the initiation of antibody production. This has also been suggested by Mond and Brunswick (1987), who demonstrated *in vitro* that IFN-γ suppressed the number of plaque forming cells to TNP-Ficoll, whereas addition of IFN-γ neutralizing monoclonal antibodies enhanced the response to TNP-Ficoll. Preliminary *in vivo* studies of our group, in which mice were treated with IFN-γ neutralizing antibodies, suggest that IFN-γ is one of the down-regulating factors in TI-2 immune responses. Identification of other cytokines involved in regulation is currently under investigation.

In summary, TI-2 antigens induce production of cytokines by T-cells. Therefore, despite their classification as T-independent, T-cells are involved in the response against TI-2. If these cytokines are an absolute requirement for these responses is not known yet. If they are, the occurrence of TI-2 responses in nude mice may be explained by cytokine production by macrophages or natural killer cells.

MIGRATION OF AFC DURING TI ANTIBODY RESPONSES

As we hypothesized earlier (Van Rooijen et al., 1986b), there is a single migration pathway for antibody forming cells directed against different types of antigens. This is reflected for instance in the localization pattern of specific AFC in a TI-2 response. To illustrate this, one can best compare the TD response against TNP-KLH (keyhole limpet hemocyanin) shown in figure 3e with the TI-2 response against TNP-Ficoll shown in figure 3f. Both figures represent rat experiments but similar, though less clear, images were obtained in the mouse as shown in figures 3d and 3i, respectively. Basically, the AFC are mainly found around the terminal arterioles and in the outer PALS, irrespective of the antigen used.

In a typical TI-1 response the localization pattern is expected to be identical to that of Ficoll, as Ficoll is a model antigen lacking only the lipid-A component. However, due to the drastic effects of lipid-A on the migration of lymphoid and non-lymphoid cells (as described in detail by Groeneveld et al., 1986), exact localization patterns are difficult to determine in a bona fide TI-1 response. For this reason we trinitrophenylated both normal lipopolysaccharide (LPS) and the detoxified (no lipid-A) version and observed a localization pattern for detox-LPS specific AFC, identical to that for other antigens (Claassen et al., 1986c). One can easily imagine that cell-cell interactions are even more difficult to study in a TI-1 model *in vivo*. However, the direct and polyclonal activation of B-cells by TI-1 antigens all but excludes the importance of the microenvironment and cell-cell interactions in this response.

ISOTYPES PRODUCED DURING TI ANTIBODY RESPONSES

An important difference in the humoral response against type 1 and type 2 TI antigens is the isotype of the antibodies produced. In a typical TI-1 response, mainly IgM is found, whereas in a typical TI-2 response IgM, IgG3 and IgG1 are found in the mouse, and IgG4 and IgG1 in man are found. In a splenic autotransplantation and regeneration model in mice, we recently showed that the isotype of the antibodies formed in a typical TI-2 response could be at least partly regulated by marginal zone macrophages. This was expressed in a temporary inability (up to ten weeks) of the animals or autotransplants to produce IgG1 antibodies directed against the TI-2 antigen. Upon return of the marginal zone macrophages this capability was fully restored (Claassen et al., 1989; 1991a).

2.6 FOLLICULAR TRAPPING OF ANTIGENS

MECHANISM OF FOLLICULAR TRAPPING

After the first administration of a TD antigen, a primary humoral immune response is initiated and immune complexes are formed as soon as antibodies appear in the circulation (Klaus et al., 1980). Immune complexes are also formed if specific antibodies against the antigen are already present in the circulation when the antigen is administered e.g. for the generation of a secondary response. In such cases the distribution of the antigen is altered when compared to uncomplexed antigen. Antibody-complexed antigens are ingested by macrophages as we discussed for non-antibody complexed antigens, but especially phagocytosis of small and soluble antigens will be facilitated in comparison with their non-opsonized counterparts. A small proportion of the antibody complexed antigens are trapped in the follicle centers on the cell processes of the FDC and retained for long periods of time (Tew et al., 1980) via Fc receptors (Nossal et al., 1968) and complement factor C3 receptors (Klaus and Humphrey, 1986).

A longstanding controversy with regard to follicular trapping is the possible cell-mediated transport of immune complexes from the marginal zone to the follicles. As discussed previously (Laman et al., 1990a), many authors have claimed that different cell types, including lymphocytes, macrophages and migrating FDC precursors transport immune complexes. However, several other lines of evidence (including studies with immune complexes and immunologically inert coal particles) indicate that migration of immune complexes may be passive, by simply following the bloodstream. We have shown that *in vivo* elimination of splenic macrophages and a large proportion of marginal zone B-cells does not influence trapping of peroxidase anti-peroxidase complexes, indicating that these cell types are not required for transport of immune complexes. In addition, we found that remnants of dead macrophages localized to the follicles (Laman et al., 1990a). Although it could be argued that antibodies directed against self-antigens can mediate trapping, it is unlikely that antibodies against macrophages are present. This implies that these cell remnants may localize here without being transported by cells, and that they are retained without involvement of antibodies. We can not exclude, however, that macrophage remnants activate or carry complement components, mediating retention in the follicle.

In summary, compounds may remain trapped in lymphoid follicles by four mechanisms: 1. By binding with specific antibodies and subsequent binding to the Fc-receptors of the FDC. Antibodies may be present as a result from a previous contact with the antigen, as cross-reactive antibodies or as 'background-housekeeping' antibodies (Bos, 1990). However, complement depletion studies point

out the requirement for complement activation (Klaus and Humphrey, 1986). It is not clear whether Fc-receptors alone are able to retain all immune complexes. 2. By binding with specific antibodies, followed by complement activation and subsequent binding to the Fc- and C3-receptors of the FDC and of C3-receptors of germinal center B-cells. 3. By direct activation of complement, without involvement of antibodies, and subsequent binding to C3-receptors of the FDC. 4. By deposition in the follicular web by the bloodstream, without involvement of specific antibodies or complement. We will use the term 'follicular trapping' to cover all four mechanisms described above.

FUNCTIONS OF IMMUNE COMPLEXES TRAPPED IN LYMPHOID FOLLICLES

It is thought that antigen which is preserved extracellulary in an undegraded form as immune complex on the FDC has a crucial role in the generation of B-memory cells in the follicles (Klaus et al. 1980; Van Rooijen, 1980). The free antigenic determinants in such trapped complexes would select antigen-specific precursors of B memory cells, resulting in the development of antigen-specific memory. Klaus and Humphrey (1977) demonstrated that complexes formed at equivalence of antigen and antibody or in slight antigen excess are far more effective than antigen alone in generating memory. C3 is required for the localization of complexes within splenic lymphoid follicles and the capacity of immune complexes to prime B-cell memory is abrogated by depriving mice of C3.

In addition, it has been suggested that the exposure and obscuring of epitopes present in immune complexes may fine-tune the titer of circulating antibodies (Tew et al., 1980). Evidence has been obtained from adoptive transfer studies that the continuous presence of antigen, possibly as immune complexes in the follicles, is required for the maintenance of B-cell memory (Gray and Skarvall, 1988; Colle et al., 1988). It has also been demonstrated that follicular immune complexes are involved in idiotypic regulation mechanisms (Klaus, 1978). Recent electronmicroscopic and *in vitro* evidence (reviewed by Szakal et al., 1989) indicates that FDC may release immune complex-coated beads, termed 'iccosomes'. These iccosomes are ingested by antigen-specific germinal center B-cells and tingible body macrophages, processed, and subsequently presented to T-cells, which then start to produce IL-2.

TRAPPING OF TI-ANTIGENS

Humphrey (1981) found that hapten-conjugated polysaccharides (TI-2 antigens) were poorly degradable and persisted for long periods of time in the circulation. IgM anti-TNP responses after immunization with TNP-Ficoll persisted for

at least 9 weeks. We found that shortly after injection of polysaccharide-conjugates, antigen is retained in relatively large quantities in the follicles, disappearing gradually but remaining detectable up to 21 days after immunization (figure 3h)(Van den Eertwegh et al., submitted). This specific localization may be explained by direct activation of the alternative complement activation pathway by polysaccharides (Griffioen et al., 1991), or by complex formation of antigen with 'background-housekeeping antibodies' (Bos, 1990) which subsequently activate the classical complement activation pathway. In both mechanisms complement is active.

Complement depletion studies have demonstrated that antibody responses against TI-2 antigens were only impaired when low doses of antigen were used (Matsuda et al., 1978). Taken together, these results suggest that follicular trapping could play a role in the induction of TI-2 immune responses. When high doses are administered, the concentration of the antigen may be sufficient for the direct activation of B-cells, and requires no antigen presentation by the FDC. Later on in the immune response, trapped antigen is still present on the FDC, which may be sufficient for sustained stimulation of antigen-specific B-cells, leading to a persistence of adequate serum levels of antigen-specific antibodies.

In contrast to TD antigens, activation of B-cells in TI immune responses requires no processing step of the antigen. This may explain the different roles of antigen retained by follicular dendritic cells in TD and TI immune responses. In case of TI-2 immune responses, antigen localized in the follicles may play a role in the induction and continuation of humoral immune responses, whereas in TD immune response immune complexes are active in the development of memory B-cells.

2.7 TERMINATION OF THE HUMORAL IMMUNE RESPONSE

Two major aspects of the termination of the humoral immune response have only recently been studied in more detail *in vivo*, viz. downregulation and memory formation. Downregulation of the immune response probably occurs through a combination of a variety of mechanisms including regulator cells, idiotype-anti-idiotype interactions and apoptosis, which are discussed below.

REGULATOR CELLS

Several cell types can produce cytokines which may downregulate the humoral immune response: this has already been discussed above.

T-cells bearing Lyt-2, a marker for the putative T-suppressor cell, are mainly found among the resting T-cells of the inner PALS and to a lesser extent in the marginal zone and red pulp (indicating that this may be migrating cells), but seldomly in the follicles (cf. Van Ewijk and Nieuwenhuis, 1985). In view of this

localization pattern which does not overlap with the compartments we identified for T-B interactions (as discussed, this is most notably the outer PALS), a major role for Lyt-2 positive cells in suppression of humoral responses in the spleen is not directly evident.

Recent *in vitro* studies have shown that human class I-restricted T-cells (Barnaba et al., 1990) and murine class II-restricted (Shinohara et al., 1988) antigen-specific T-cells can be generated which selectively kill antigen-specific B-cells. This would provide an effective downregulating mechanism for the humoral immune response. The *in vivo* relevance of these findings has yet to be established, however.

IDIOTYPE-ANTI-IDIOTYPE INTERACTIONS

Anti-idiotype antibodies bind to unique immunoglobulin (Ig) variable (V) region determinants of antibodies, designated the idiotype or Id. This property of anti-Ids can identify clonally related AFC populations, discriminating between AFC with subtle V region differences. We employed a two color direct double immunohistochemical staining to identify B-cells producing antibodies specific for the hapten arsonate (Ars), using Ars-antigen-enzyme conjugates and anti-id antibodies. We observed extensive idiotypic heterogeneity within clustered AFC in spleens, demonstrating close physical association between idiotypically related, but not identical, B-cells (Brown and Claassen, 1988;1989;1990). Such co-localizing AFC may either be clonally derived, having experienced V region mutations causing differences in Id expression, or they migrate in close proximity. The latter possibility seems the most likely explanation, since tightly clustered antigen-specific AFC with no detectable clonal (idiotypic) relationship were also often detected. Alternatively, these AFC may co-localize after cessation of migration upon exposure to antigen.

Self-antigen specific antibodies can be detected in the cytoplasm of AFC immunohistochemically by using antigen-enzyme conjugates for detection. In this way, the specificity of the antibodies can be detected before they have complexed with the self-antigen in the blood or in tissues. With this antigen-enzyme approach we have identified auto-anti-allotype specific AFC antibodies in spleens and bone marrow of neonatally allotype-suppressed rabbits (Claassen and Adler, 1988a; Adler and Claassen, 1989). Using a modification of this technique, we have also identified auto-anti-id producing AFC in spleens of mice after secondary immunization with antigens that stimulate high production of idiotype. A sequential staining procedure allowed the detection of both Id and anti-Id (which are crossreactive) producing AFC in the same tissue. Id and auto-anti-Id were commonly found in close proximity around the terminal arterioles and some in the outer PALS. This localization is similar to that of other AFC, suggesting that AFC may actively downregulate each other at the microenvironmental level (Brown and Claassen, 1991).

APOPTOSIS

The process of programmed cell death, apoptosis, probably executed by macrophages through an interaction of their vitronectin receptor and the target cell (Savill et al., 1990), may be critically involved in the downregulation of the humoral immune response. Apoptosis has been described to be responsible for the self antigen-driven deletion of auto-reactive cells during T-cell development (Jenkinson et al., 1989). Furthermore, Liu et al. (1989) have shown that the deletion of B-cells expressing immunoglobulin of low affinity, is also antigen-driven and probably occurs in the germinal centers by apoptosis. Although these studies are concerned with selection and affinity maturation in the early phases of the response it is quite well possible that apoptosis is involved in termination of the response also. However, on the basis of our localization studies, we predict that this phenomenon will not occur in the germinal centers but around the terminal arterioles and in the outer PALS. There is as yet no experimental evidence for this and further experiments are clearly needed in this area.

MEMORY FORMATION AND MAINTENANCE

B-cell memory formation and maintenance are of fundamental scientific interest but also have far-reaching practical implications for the design and application of vaccines. As discussed above, antigen is stored in the form of immune complexes for prolonged periods of time on FDC in the follicles. These complexes seem to be involved in the generation of B-cell memory. This identifies the follicles as the site for the generation and maintenance of memory. Evidence has been obtained that the germinal center is an important site for selection and differentiation of virgin B-cells, including heavy chain class switching (Kraal et al., 1982) and hypermutation (Apel and Berek, 1990). The process of affinity maturation may be directly linked to memory formation.

Jacob et al. (1991) have proposed that the distinct populations of AFC arising in the germinal center and the periphery of the PALS after primary immunization with a hapten-protein conjugate may use distinct differentiation routes. Germinal center AFC would arise from single founder cells that follow a process of somatic hypermutation and selection, resulting in intraclonal competition for antigen among mutated sister cells. Foci of AFC in the PALS would reflect interclonal competition between unrelated clones expressing unmutated V region gene segments.

A major question related to B-cell memory is how memory B-cells attain their prolonged lifespan. Recent evidence indicates that the proto-oncogene Bcl-2, an inner mitochondrial protein, is able to block programmed cell death of B-cells and extends the persistence of both immunoglobulin secreting cells and memory cells

(Nuñez et al., 1991; Liu et al., 1991).

In view of findings described by Liu et al. (1988), the follicle is probably not the only compartment involved in memory formation, as these authors could also detect hapten binding B-cells in the marginal zone after immunization with a haptenated TD antigen. These cells were designated as memory cells because of their capacity to mount a secondary immune response. However, it should be noted that these experiments were performed in the rat and that no such cells could be demonstrated in mice (our unpublished results; Liu et al., pers. comm.). The fact that these hapten-binding cells in the marginal zone could also be demonstrated after immunization with haptenated TI antigens (Zhang et al., 1988) and that they were dependent on antigen persistence (Colle et al., 1988) suggests that the marginal zone is a serious candidate in harbouring or recruiting memory B-cells. This would be advantageous with respect to the optimal microenvironment provided here for the encounter of these cells with antigen and subsequently with the cell-cell interactions required for a fast secondary immune response.

The studies of Zhang et al. (1988) and Colle et al. (1988) provided new information for the longstanding discussion on memory formation by TI-antigens. It seems clear now that some TI-antigens can evoke B-cell memory. This may be dependent on the TI-antigen used, as we have not been able to find evidence for B-cell memory against TNP-Ficoll (unpubl. results).

In conclusion, recirculating memory B-cells preferentially migrate into germinal centers (Vonderheide and Hunt, 1990) and the marginal zone harbours relatively sessile antigen-binding cells which could be responsible for secondary responses. As antigenic stimulation has been implicated in altering B-cell migration in the spleen (Sprent and Lefkovitz, 1976; Gray et al., 1984b), it is possible that memory B-cells generated in lymph node germinal centers stop recirculating when exposed to antigen captured in the spleen, causing AFC of diverse specificities to accrue around antigen depot sites (Brown and Claassen, 1991). It should be noted, however, that B-cell memory formation and germinal center generation can be generated with immune complexes made in antibody excess without the generation of a detectable immune response (Kraft et al., 1989). The mechanism underlying this phenomenon is not clear, but the authors speculate that the covering of all epitopes on the antigen by specific antibody may lead to a dissociation of the germinal center/memory pathway on the one hand, and the primary humoral response on the other.

CHAPTER 3

HIV-1 INFECTION AND THE HIV-1 SPECIFIC ANTIBODY RESPONSE

3.1 HIV-1: IDENTIFICATION AND TRANSMISSION

AIDS (acquired immune deficiency syndrome) was identified as a new disease entity in the American homosexual community in the early 1980's. Due to malfunctioning of the immune system, patients fall prey to a multitude of infectious pathogens and neoplasms. Scepticism of the general public, some public health officials and politicians, and in addition of individuals within the homosexual community frustrated early attempts to slow down the epidemic and to initiate scientific research, allowing the illness to spread to immense social cost (described in detail by Shilts, 1987). HIV-1 infection and AIDS now represent a formidable socio-economical threat, in view of the large number of infected persons worldwide (Blattner, 1991).

After the first successful isolation of the human retrovirus HIV-1 (previously also called LAV, HTLV-III and ARV) by Barré-Sinoussi et al. (1983), this virus was aetiologically linked to AIDS by a multitude of studies (Popovic et al., 1984; Gallo et al., 1984). HIV-1 belongs to the Lentivirinae subfamily of retroviruses which induce slowly progressive disease characterized by long clinical latency periods and involvement of the central nervous system. CD4-carrying lymphoid cells critically involved in immune function are target of HIV-1 infection (see below) as well as cells in the central nervous system. HIV-1 is present in body fluids like blood, semen, cervical secretions and saliva. Transmission can occur horizontally through blood-blood contact, unprotected anal or vaginal intercourse, or vertically from mother to child during pregnancy or delivery (reviewed by Blattner, 1991) It is thought that both cell-free virus and virus-infected cells can transmit infection. The initial clinical identification of AIDS, the discovery of HIV-1, its linkage to AIDS, and the question whether AIDS is a new disease or conversely an ancient African disease, have been extensively and eloquently reviewed by Grmek (1990).

The years following the discovery of HIV-1 have seen an extremely rapidly growing body of knowledge concerning the biology of the virus, including genomic organization and regulation, genetic variability and biochemical composition. Comparatively, understanding of *in vivo* host-virus interactions has lagged behind, due to the greater complexity of such studies and the lack of an adequate (small) animal model. As to this day, the actual mechanisms underlying immunopathogenesis of AIDS are largely unknown. To place the work presented in this thesis in the broader context of HIV-1 infection, a condensed background on HIV-1 and AIDS is given below. Where possible, the reader is referred to recent reviews for extensive discussion and additional original publications.

3.2 VIRAL STRUCTURE

The genome of HIV-1 consists of two single strands of RNA, containing the typical retrovirus genes *gag*, *pol* and *env*, which encode the proteins of the core nucleocapsid, the viral enzymes and the membrane proteins, respectively. The genome contains at least six additional genes, of which the functions are not all clear yet (table 2). Discussion of the molecular biology and biochemistry of HIV-1 is beyond the scope of this thesis: these subjects are reviewed extensively by Greene (1991) and Vaishnav and Staal (1991).

TABLE 2. HIV-1 GENE PRODUCTS AND THEIR FUNCTIONS

GENE	PROTEIN	FUNCTION
STRUCTURAL		
gag	p55/53	precursor
	p7	binds to the genomic RNA
	p9	present in nucleoid core
	p17	associated with inner surface of lipid bilayer
	p24	main constituent of inner shelf of nucleocapsid
pol	p51/p66	subunits of reverse transcriptase: RNA to DNA transcription
	p10	protease: cleavage events during final budding
	p32	endonuclease, integrase: integration of viral DNA-duplex into host genome
	p13	ribonuclease: partial degradation RNA-template
env	gp160	precursor
	gp120	external membrane protein mediating CD4 binding and membrane fusion
	gp41	transmembrane protein involved in fusion
REGULATORY		
vif	p23	infectivity factor
vpu	p16	promotion of efficient release of budding virions from cell surface
vpr	p15	unclear: weak transcriptional activator
tat	p14	transactivator of all viral genes
rev	p19	post-transcriptional regulator of structural gene products
nef	p27	unclear: previously implicated in downregulation of gene expression and latency

HIV-1 shows high genetic variability throughout the genome but most notably in the envelope region. The core proteins are more conserved. Field isolates consist of a mixture of highly related viral variants (cf. Goudsmit et al., 1991), indicating that *in vivo* variation of the virus may occur. This mechanism of antigenic drift may be promoted by immune pressure exerted by antibodies and cellular responses, and is favoured by the high mutation rate of HIV-1. Emerging new variants can escape

immune surveillance effected by antibodies (Albert et al., 1990; reviewed by Nara et al., 1991) or cytotoxic cells (Takahashi et al., 1989).

The complete virion structure of HIV-1 is shown in figure 4. The *env*-precursor protein gp160 is intracellularly cleaved to gp41 and gp120. gp41 is a transmembrane molecule (both in the virus particle and in virus-infected cells) that contains a putative fusion sequence at the hydrophobic N-terminus (Gallaher, 1987). gp41 is associated with gp120 through non-covalent bonds. This association is weak allowing HIV-1 to shed gp120-molecules (Gelderblom et al. 1985; Schneider et al., 1986). Soluble CD4, the cellular receptor for HIV-1 (see below), can release gp120 from gp41 on the membrane of the virion (Moore et al., 1990). Both gp41 and gp120 are present as oligomers, but dependent on the experimental approach used dimers, trimers or tetramers composed of two dimers are found (reviewed by Gelderblom, 1991). Morphological studies indicate that gp120 is present on the viral membrane as a trimer (Gelderblom, 1991).

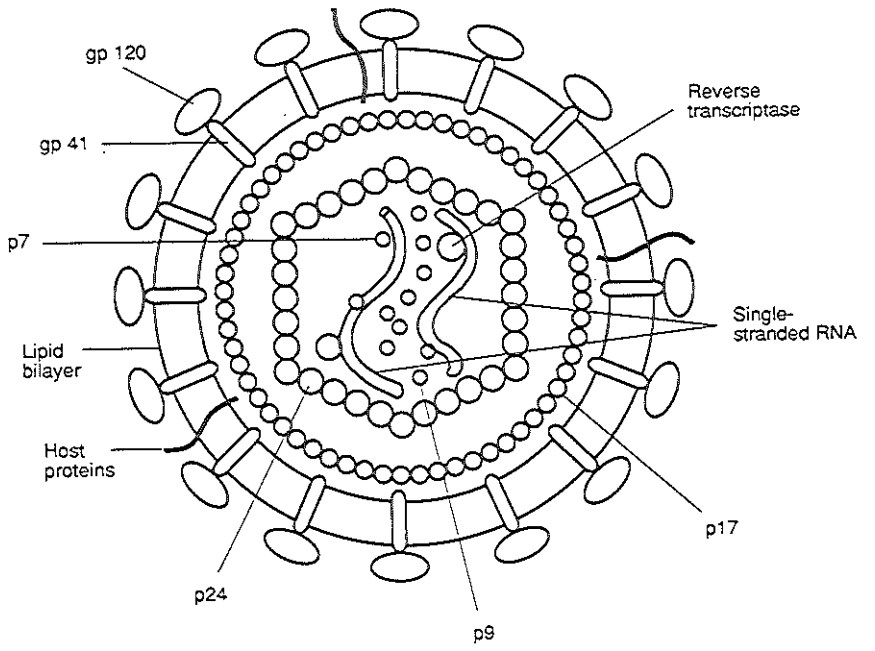

FIGURE 4. STRUCTURE OF HIV-1

The structural proteins of the envelope glycoprotein and the nucleocapsid are shown. In the nucleocapsid, the two strands of RNA and the reverse transcriptase are drawn. As the lipid bilayer is derived from the membrane of the host cell during the budding process, host proteins can be present in the virion. Adapted from Greene (1991).

In the gp120 moiety of the *env*-gene, five variable and six relatively conserved regions have been identified. gp120 is highly glycosylated with N-linked carbohydrates. The third variable domain of gp120, V3 (also designated as V3-loop, V3-domain or principal neutralizing determinant: PND), has been extensively studied in view of its role in evoking neutralizing antibodies (see below). The V3-domain consists of 36 amino acids (in the case of isolate IIIB) which form a loop as a result of disulfide-bonding of cystein residues at positions 303 and 338 (Gregory et al., 1990)(see also figure 5). V3 is essential for infectivity (Kowalski et al., 1987) and plays a role in virus-cell and cell-cell fusion (Freed et al., 1990). It has been hypothesized that proteolytic cleavage of V3 by cell surface or endosomal proteinase is necessary for fusion to occur and that neutralizing antibodies directed against V3 prevent this cleavage (Clements et al., 1991). The presence of positively charged amino acid residues may promote the fusion function of V3 (Callahan et al., 1991). V3 has also been implicated as a primary determinant of macrophage tropism of HIV-1 strains (cf. Takeuchi et al., 1991; Hwang et al., 1991).

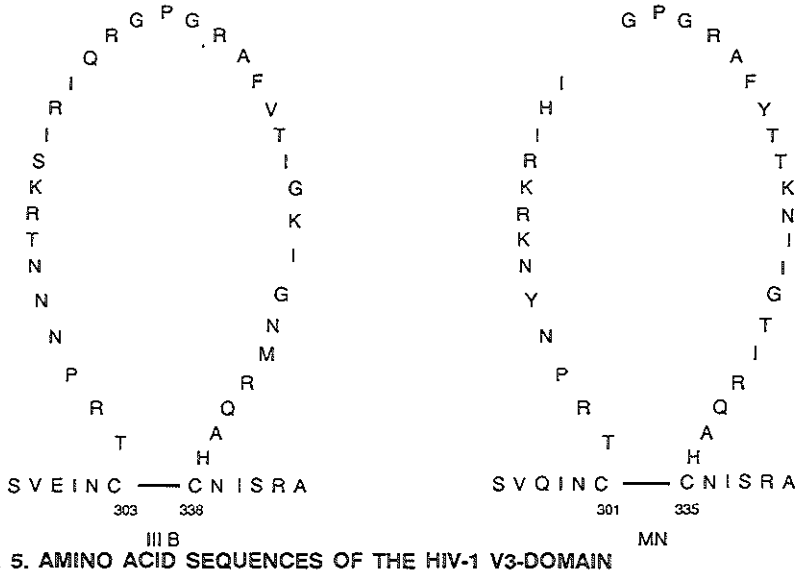

FIGURE 5. AMINO ACID SEQUENCES OF THE HIV-1 V3-DOMAIN

The amino acid sequence of the third variable domain of gp120 of two HIV-1 variants is shown. IIIB is the intensively studied first isolate of HIV-1, which seems to have an exceptional primary amino acid sequence (Barré-Sinoussi et al., 1983; Ratner et al., 1985). Sequences similar to those contained in the MN V3-domain are much more prevalent in naturally occurring infections (LaRosa et al., 1990; Zwart et al., 1991). The V3-domains are drawn to emphasize differences in primary amino acid sequence: note the absence of the Q (glutamine) and R (arginine) residues in MN. The figure does not reflect the three-dimensional folding of the V3-domain, which is not known at present.

3.4 THE INFECTIOUS PROCESS AND DISEASE PROGRESSION

The tropism for CD4-carrying cells is conferred to HIV-1 by gp120 (Dalgleish et al., 1984; Klatzmann et al., 1984; Maddon et al., 1986). The gp120 binding site of CD4 is a relatively short segment that spans amino acids 40-60 of the first domain, which is structurally related to the second complementarity determining region of immunoglobulin light chain (CDR2). The CD4 binding site on gp120 is discontinuous, involving five regions which are probably brought together by intramolecular folding to interact with the relatively small site on CD4. In addition, oligomerization of gp120 molecules may be involved in CD4-gp120 interaction. The affinity of the gp120-CD4 interaction depends largely on the virus strain and the Km has been estimate to be in the order of 4×10^{-9} M (reviewed by McDougal et al., 1991).

It is thought but not yet substantiated that the interaction of gp120 with CD4 leads to exposure of the fusion peptide in gp41, allowing fusion to take place. Internalization of CD4 is not required for infection which can occur through virus-cell fusion (Bedinger et al., 1988; Maddon et al., 1988). Other as yet unidentified cell structures may be involved in virus entry. The formation of syncytia (multinucleated giant cells) *in vitro* by fusion of HIV-1 infected cells with other CD4-expressing cells (Sodroski et al., 1986; Lifson et al., 1986) is thought to involve the same mechanisms as virus-cell fusion.

HIV-1 is able to infect cells in the central nervous system that lack demonstrable expression of CD4. This pathway may involve galactosyl ceramide as a receptor (Harouse et al., 1991). The tropism of HIV-1 for cells of the central nervous system is related to the neurological disorders seen after HIV-1 infection (reviewed by Price et al., 1988).

NUMBER OF INFECTED CELLS AND VIRAL LOAD

The number of latently and actively HIV-1 infected cells *in vivo* is a matter of debate. *in situ* hybridisation studies (Harper et al., 1986) indicated that 1×10^5 lymph node cells and PBMC (i.e. lymphocytes and monocytes) of AIDS patients contain HIV-1 mRNA, reflecting active replication. PCR-studies showed that asymptomatic patients have one provirus molecule per 6,000 to 8,000 PBMC, while AIDS patients have one provirus molecule per 700 to 3,300 PBMC (Simmonds et al., 1990). Schnittman et al. (1989) have provided evidence that the CD4+ T-cell is the principal reservoir of HIV-1 in PBMC. In AIDS patients, HIV-1 DNA was detected in more than 1 per 100 PBMC, but HIV-1 expression was found in about 1 per 1,000 PBMC. Schnittman et al. (1991) have also demonstrated that active viral expression, as evidenced by HIV-1 mRNA, occurs at all stages of HIV-1 infection. Ho et al. (1989) have shown that infectious HIV-1 can be recovered from plasma and PBMC of all

asymptomatic and symptomatic individuals tested, with higher titers for the latter group. In asymptomatics, 1 in 50,000 PBMC harbored HIV-1, for symptomatics this ratio was 1 in 400. It has been suggested that a relation exists between increasing viral burden and disease progression (cf. Schechter et al., 1991).

A multitude of signals can lead to activation of latent HIV-1 provirus *in vitro* (reviewed by Rosenberg and Fauci, 1991). Which factors govern activation of HIV-1 latently present *in vivo* is currently unknown.

HIV-1 variants differ in biological properties as defined by cell tropism (monocytes/macrophages, T-cells), growth characteristics (slowly growing to low titers vs. rapidly growing to high titers) and induction of syncytium formation (syncytium inducing vs. non-syncytium inducing: SI/NSI). It has been hypothesized (Miedema et al., 1990) that after initial infection, highly virulent SI strains are effectively suppressed by the immune system. Variants growing at low rate persist, possibly present in cells of the monocyte/macrophage lineage. The infidelity of the reverse transcriptase leads to constant generation of new variants. Virulent variants take advantage of the breakdown of the immune system that may result from depletion of T-memory cells and/or dysfunction of antigen presenting cells. The outgrowth of virulent strains eventually induces AIDS. Antigen presenting cells such as macrophages and dendritic cells can also be infected with HIV-1, interfering with antigen presentation to B- and T-cells.

It has been shown that dendritic cells in the peripheral blood of infected persons can be infected by HIV-1, leading to dysfunction in antigen presentation to T-cells, and to depletion of the dendritic cell population (Macationa et al., 1990). Infection of the dendritic Langerhans cells in the epidermis is a matter of controversy but a recent extensive study showed that these cells are not principal reservoirs of the virus (Kalter et al., 1991).

The significance of syncytium formation by HIV-1 infected cells (Lifson et al., 1986; Sodroski et al., 1986) for disease progression is unknown, as syncytia are seldomly found in lymphoid tissues or peripheral blood. It cannot be excluded that the *in vivo* half life of syncytia is short, limiting the chance of detection.

HIV-1 AND AUTOIMMUNITY

Several regions of HIV-1 showing molecular mimicry with host proteins have been identified, which may lead to direct functional mimicry or indirect immunological mimicry (reviewed by Bjork, 1991). Functional mimicry is thought to occur as a result of a homology between HIV-1 and neuroleukin, a factor promoting neuronal proliferation. In this way, HIV-1 may inhibit neuronal growth. Immunological mimicry may result from homologies between HIV-1 and three regions of HLA class II, a site in HLA class I (Grassi et al., 1991) and sites in human immunoglobulin IgA2, IgG2

and IgG4. A recent study (Kion and Hoffmann, 1991) has shown that immunization of mice with lymphoid cells of another murine strain results in production of antibodies against gp120 and p24, demonstrating the HLA/MHC properties of HIV-1 proteins. Auto-antibodies against lymphocyte surface antigens, HLA-antigens and immunoglobulins have indeed been detected in HIV-infected persons (Bjork, 1991). These antibodies may be involved in impairment of T-helper cell responses, activation of B-cells by crosslinking of surface immunoglobulin and other autoimmune reactions. Evidence has been found that immunization of rhesus macaques with a human T-cell line (C8166) devoid of virus may confer protection against subsequent intravenous challenge with SIV (Stott et al., 1991). The explanation that this protection results from homologies between HIV-1 and HLA sequences is highly speculative at this moment.

HIV-1 INDUCED HISTOPATHOLOGY OF THE LYMPH NODES

HIV-1 infection is accompanied by extensive histopathology of the lymph nodes. Studies of lymph node biopsies taken from infected persons at various clinical stages of disease have revealed a correlation of the histopathology with progression of the disease. A histopathological staging scheme has been developed, based on routine histology and immunocytochemical staining of lymph node sections (tables 3a and 3b)(cf. Rácz et al., 1986; Öst et al, 1989). The morphology of the follicles is crucial to this staging scheme.

Initially, many hyperplastic follicles are found. In the subsequent stages the follicles are progressively destructed, eventually leading to the complete loss of lymph node structure, with a concurrent loss of lymphoid elements. A prominent feature of HIV-1 related histopathology of the lymph nodes is the destruction of the follicular dendritic cell network. As FDC are critically involved in B-cell responses (see chapter 2), their destruction may be related to B-cell defects seen in HIV-1 infected persons. There are as yet no satisfactory explanations for the plethora of pathological events occurring in the lymph nodes. It is paramount however, that the profound disorganization of the lymph nodes will be reflected in lymphoid function.

Chapter 6 of this thesis addresses the destruction of FDC and HIV-1 specific B-cell responses *in situ*. HIV-1 infection also correlates with histopathology of the spleen and thymus. These organs have been far less well studied as only autopsy material and no biopsy material is usually available, and they will not be discussed here.

3.5 B- AND T-CELL FUNCTION DURING HIV-1 INFECTION

HIV-1 infection evokes cellular immune responses (reviewed by Rivière, 1991; Nixon and McMichael, 1991): 1. ADCC directed against the viral envelope is exerted by natural killer (NK) cells armed with antibody. 2. Cytotoxic responses directed against epitopes of *gag*, *pol*, *nef* and *env* are exerted by CTL. These responses can be MHC class I or class II-restricted. 3. Suppression of viral replication is exerted by an as yet uncharacterized diffusible factor secreted by CD8+ T-cells. The significance of the different cellular immune responses in limiting viral spread and progression of disease remains to be elucidated.

One of the hallmarks of HIV-1 infection, the disappearance of CD4+ T-cells, is not understood. Several mechanisms have been proposed, including lytic effects of budding virions, toxic effects of accumulating viral mRNA or proteins, syncytium formation, and cytolytic attack of uninfected CD4+ T-cells that display processed gp120 determinants by specific CTL. It is clear that solving this issue is of crucial importance to understand HIV-1 induced pathology and design adequate therapy.

T-cell functional defects precede the decline in CD4+ T-cell numbers (reviewed by Miedema et al., 1990; Shearer and Clerici, 1991). First, responses to recall antigens are lost, followed by those to alloantigens and finally those to mitogens. These qualitative defects may be the result of one or more of the following factors: failure to produce IL-2, selective loss or inactivation of T-memory cells, defects in antigen presenting cells or presence of inhibitory soluble factors like autoantibodies (see below), gp120, *tat* protein or transforming growth factor beta (Shearer and Clerici, 1991).

B-CELL FUNCTION IN HIV-1 INFECTION

A number of B-cell abnormalities has been reported to occur in HIV-1 infected individuals (reviewed by Lane and Fauci, 1985; Amadori and Chieco-Bianchi, 1990). These abnormalities include changes indicative of hyperreactivity but also of immaturity/non-responsiveness. Polyclonal hypergammaglobulinemia is found in asymptomatics and in symptomatics as evidenced by increased immunoglobulin levels, including IgD, increased numbers of B-cells secreting immunoglobulins *in vitro*, and increased production of immunoglobulins *in vitro*. HIV-1 specific antibodies form a major component in this response as B-cells from seropositive individuals spontaneously produce anti-HIV-1 antibodies *in vitro* (e.g. Amadori et al., 1988).

In vitro stimulation of B-cells with pokeweed mitogen (PWM) and/or Epstein-Barr virus has been used to detect HIV-1 specific antibody production in seronegative individuals (Jehuda-Cohen et al., 1990) and to discriminate between maternal antibodies and antibodies produced by children born to HIV-1 infected

TABLE 3a. HIV-RELATED LYMPHADENOPATHY: HISTOPATHOLOGICAL CRITERIA

FOLLICLE	INTERFOLLICULAR SPACE
PHASE I. IRREGULAR FOLLICULAR HYPERPLASIA (IN PGL)	
- follicles increased and enlarged - bizarre follicle shapes - centroblasts dominant - multiple starry sky macrophages - regular fibre structure - mantle zone intact - high mitosis rate	- hypercellularity - predominant cell types: lymphocytes, macrophages - individual epithelioid and giant cells - lymphoblasts, immunoblasts - small capillary and venule proliferations - nests of IDC
PHASE II. BEGINNING OF FOLLICULAR DESTRUCTION (IN PGL AND ARC)	
- large irregularly formed follicles - depletion of centroblasts, FDC and starry sky macrophages - irregularly formed and broken mantle zone with monocytoid B-lymphocytes - incipient dissolution of the reticular fiber structure	- reduction of cell density - small groups of epithelioid cells - monocytoid B-lymphocytes - reduction of lymphoblasts and immunoblasts - nests of IDC - angioneogenesis
PHASE III. PROGRESSIVE FOLLICULAR DESTRUCTION (IN ARC AND AIDS)	
- large, indistinctly demarcated, fading ('exploding') and/or shrinking follicles - loss of mantle zone - depletion of monocytoid B-lymphocytes - marked reduction of germinal center cells and starry sky macrophages - marked increase in lymphocytes - incipient vascularization of large fading follicles	- increased angioneogenesis - reduction of lymphocytes and IDC - increased number of macrophages - depletion of monocytoid B-lymphocytes
PHASE IV. FOLLICULAR INVOLUTION AND ATROPHY (IN ARC AND AIDS)	
- hyalinisation of shrinking follicles - vascularization of fading follicles - follicles occupied almost exclusively by lymphocytes	- marked increase in angioneogenesis - further reduction of lymphocytes and IDC - marked increase in macrophages and plasma cells
PHASE V. COMPLETE LOSS OF LYMPH NODE STRUCTURE (IN AIDS)	
- 'naked' connective tissue structure - diffuse distribution of macrophages, plasma cells and scattered lymphocytes - hyaline plaques and foci of vascular proliferation as follicle remnants - occasionally extreme angioneogenesis	

TABLE 3b. HIV-RELATED LYMPHADENOPATHY: IMMUNOHISTOCHEMICAL CRITERIA

FOLLICLE	INTERFOLLICULAR SPACE
PHASE I. IRREGULAR FOLLICULAR HYPERPLASIA (IN PGL)	
- dominance of CD19+ cells (B-cells) - all germinal center cells express HLA-DR - intact network of KiM4+ FDC - marked proliferation of FDC and lymphoid cells in the germinal center (Ki67) - fewer CD4+ lymphocytes - numerous CD68+ macrophages - viral proteins in macrophages, FDC and some lymphocytes	- onset of inversion of CD4/CD8-ratio (initially due to increase in CD8+-cells) - numerous CD68+ macrophages - increased KiM1+ and CD1+ IDC - proliferation of lymphoid cells and IDC - viral proteins in macrophages, IDC and some lymphocytes - numerous HLA-DR+ T-lymphocytes - numerous CD4-, CD8-, CD3+ cells
PHASE II. BEGINNING OF FOLLICULAR DESTRUCTION (IN PGL AND ARC)	
- reduction of B-cells - initial disintegration (tearing) of the FDC network - increase in CD4+, CD8+ and HLA-DR+ lymphocytes - decline in proliferation activity - viral proteins as in Phase I - numerous HLA-DR+, CD4+, CD8+ lymphocytes	- progressive decrease in CD4/CD8 ratio (decrease in CD4+ cells) - decline in proliferation activity - increase in macrophages (CD68+) and AFC - increase in endothelium - numerous CD4-, CD8-, CD3+ cells
PHASE III. PROGRESSIVE FOLLICULAR DESTRUCTION (IN ARC AND AIDS)	
- marked reduction of CD19+ cells - diffuse depletion of FDC - increase in CD8+ cells, few CD4+ cells - residual proliferative activity - viral protein containing cells	- marked further reduction in CD4/CD8 ratio - increase in macrophages (CD68+) and AFC - decrease in viral protein containing cells - moderately numerous HLA-DR+ lymphocytes
PHASE IV. FOLLICULAR INVOLUTION AND ATROPHY (IN ARC AND AIDS)	
- germinal center cells almost entirely replaced by CD8+ cells - macrophages and isolated CD4+ lymphocytes	- extreme reduction or absence of CD4+ cells - sparse HLA-DR+ lymphocytes - viral protein containing cells
PHASE V. COMPLETE LOSS OF LYMPH NODE STRUCTURE (IN AIDS)	
- very high numbers of macrophages and AFC - scattered CD8+ lymphocytes - few CD4+ lymphocytes - viral protein containing cells	

mothers (Pahwa et al., 1989; De Rossi et al., 1991). However, mitogen stimulation can also suppress HIV-1 specific antibody production *in vitro* by a mechanism that is dependent on the presence of CD8+ T-cells (De Rossi et al., 1991). Terpstra et al. (1989) have found that B-cell unresponsiveness to PWM is seen from seroconversion on. This defect seems to be intrinsic to the B-cell, and is not due to reduced CD4+ T-cell function. Although the numbers of circulating B-cells are normal, an increase in activated B-cells as defined by expression of the transferrin receptor, and a decrease of resting B-cells (Leu-8 positive) is seen in seropositive individuals. AIDS-patients show an increase in circulating immature B-cells (CALLA-positive: CD10)(Martinez-Maza et al., 1987).

Conflicting data have been obtained with regard to immunization of HIV-1 infected individuals. Discrepancies may be related to limited numbers of subjects studied and differences in the experimental design. In general, antibody responses to immunization with pneumococcal antigens are impaired in patients with AIDS-related complex (ARC) or AIDS, and somewhat less impaired in symptomless HIV-infection. Pre-immunization titers are also lower in both symptomatics and asymptomatics. Reduced responses have also been seen for T-dependent recall and neo-antigens (reviewed by Pinching, 1991).

This broad spectrum of B-cell abnormalites occurring during HIV-1 infection is poorly understood thus far. Although some studies show that B-cell lines can be infected with HIV-1 *in vitro* (cf. Dahl et al., 1990), there is no evidence for infection of B-cells *in vivo*. Schnittman et al. (1986) have shown that HIV-1 directly stimulates B-cells to proliferate and differentiate. Pahwa et al. (1985) found both stimulatory and inhibitory effects of HIV-1 proteins on B-cells. The presence of T-cells was required for the stimulatory effects (see also Yarchoan et al., 1986). It is not known whether the concentrations of HIV-1 proteins used *in vitro* in these studies represent meaningful physiological concentrations. Many groups have tried to correlate B-cell activation to Epstein-Barr virus (EBV) infection, but no clear relationship could be established.

IL-6, an interleukin involved in stimulation of B-cells, is induced in PBMC *in vitro* by live and inactivated HIV-1 (Nakajima et al., 1989), and IL-6 was found to be crucial for *in vitro* synthesis of total and anti-HIV immunoglobulins (Amadori et al., 1991). Increased IL-6 production was also found in HIV-1 infected persons (Breen et al., 1990), but not in the study of Amadori et al. (1991). This increased IL-6 production may be related to the signs of B-cell activation during HIV-1 infection.

Using human CD4+ T-cell clones, Macchia et al. (1991) showed that HIV-1 infected CD4+ cells can induce antigen-independent, contact dependent polyclonal B-cell activation. This is an intriguing finding that has to be explored further, and has to be shown to be operational *in vivo*.

A recent study by Berberian et al. (1991) showed that HIV-1 seropositive

individuals show a striking deficit of B-cells expressing the rearranged VH3L gene. In seronegative subjects, these cells are normally abundant in the blood and the lymphoid mantle zone, but absent from the germinal center. These data point to a maturational arrest of B-cells at the germinal center stage, but the underlying mechanism is not clear.

ANTIBODY RESPONSE AGAINST HIV-1

All HIV-1 structural and regulatory proteins evoke specific circulating antibodies in infected persons. The serologic profile after primary infection shows a variable latency period (weeks to months) followed by an IgM response which declines after some time, and a subsequent IgG response, which is sustained. Antibodies to p24 (Lange et al., 1986), reverse transcriptase and the viral protease seem to have prognostic value, as a decline in titer precedes the development of AIDS. In general, antibody titers against all HIV-1 proteins are lower in AIDS-patients than in asymptomatics. Antibodies to gp41 are an exception to this rule as titers are comparable during different stages of the disease. An additional parameter in serology is the presence of p24 antigen: when anti-p24 antibody levels decline, a rise in circulating p24 antigen is usually seen. Formation of soluble immune complexes of p24 and anti-p24 influences the presence and detection of both p24 antigen and specific antibodies. Teeuwsen et al. (1991) have found that the number of functionally active B-cells producing p24-specific antibodies may be reduced during progression towards AIDS.

Antibodies capable of neutralizing HIV-1 infectivity or HIV-1 induced syncytium formation *in vitro* have been found in infected individuals. gp120 and most notably its third variable domain (V3) seems to be the major target for this neutralizing activity. Other neutralization epitopes have been identified on p17 and gp41 (reviewed by Goudsmit et al., 1991; Nara et al., 1991). In chapter 7 of this thesis specific antibodies to probe the role of distinct V3-sequences in neutralization are described. The V3-neutralization epitopes seem to be sequential epitopes. However, evidence has been obtained that discontinuous neutralization epitopes are present on HIV-1 gp120 (Steimer et al., 1991; Ho et al., 1991).

It should be noted here that neutralization of viral infectivity by antibody is a fully operationally defined term. Neutralization may result from simple agglutination of virions, or from obscuring the binding site of the virus from its ligand, but this latter possibility seems to be an exception. Neutralization can also result from induction of conformational changes preventing infection or conversely from the prevention of conformational changes which are required for infection (reviewed by Dimmock, 1987). The isotype of the antibody can determine the mechanism of neutralization. Therefore, the mechanism of neutralization has to be determined for

every combination of an antibody and its complementary neutralization epitope.

HIV-1 specific antibodies and complement may promote progression of the disease by enhancing cellular infection by antibody dependent enhancement (ADE) involving the Fc-receptor, and complement mediated enhancement (CDE) involving the complement receptor (Takeda et al., 1988; Robinson et al., 1988).

3.6 VACCINE DEVELOPMENT

Worldwide, considerable effort is devoted to the development of vaccines preventing AIDS. In general, a vaccine can induce sterile immunity, preventing infection most probably by induction of high levels of virus-specific antibodies, or systemic immunity, preventing disease either by antibodies, cytotoxic T-cells or both. It is unknown whether sterile or systemic immunity for HIV-1 can be achieved with a vaccine.

In theory, the following immunogens can be used: live attenuated or recombinant virus; whole inactivated virus; natural subunit products; recombinant DNA subunit products; synthetic peptides or anti-idiotypic antibodies. Conventional vaccine strategies using attenuated or inactivated pathogens will probably not be acceptable for HIV-1 as recombinatory events and the infidelity of the reverse transcriptase may generate new virulent variants. In addition, complete inactivation or removal of infectious virions from vaccine preparations can not be achieved with certainty.

Current successes in vaccine trials in primates using formalinized SIV (Murphey-Corb et al., 1990; cf. Stott et al., 1991), recombinant gp120 (Berman et al., 1990) and a combination of synthetic peptides and recombinant antigens (Girard et al., 1991) have boosted optimism regarding the development of an anti-HIV-1 vaccine. This optimism has been tempered by the finding that the cell lines used to prepare experimental vaccines may contribute to protection (Stott et al., 1991).

HIV-1 vaccine studies face several difficulties. First, it is as yet unknown whether protective immunity against HIV-1 can be evoked with a vaccine, and, if so, which immune effector mechanisms effect this protection. Second, the ideal HIV-1 vaccine should not only protect against disease, but should also evoke antibodies that prevent HIV-1 infection from ocurring at the mucosa (i.e. by local production of IgA antibodies), and HIV-1 infection occurring in the bloodstream (i.e. by circulating IgG and IgM). This issue is further complicated by the fact that HIV-1 may also be transmitted intracellularly, where it cannot be reached by neutralizing antibody. Once infection has been established, antibodies and cytotoxic cells may not be able to clear all virus from the body. Third, HIV-1 displays high genetic variability, complicating the construction of an immunogen evoking broad-spectrum immunity. As sterilizing immunity is not achieved, the vaccinated HIV-1 infected patient remains

infectious for other individuals. However, construction of a vaccine which slows down disease progression, albeit not capable of eventually preventing AIDS, would already be a major achievement.

Several studies included in this thesis address the difficulties described above. Methods developed to study HIV-1 specific antibody responses *in situ* (chapter 5) can be used to evaluate humoral responses evoked by HIV-1 infection or by candidate vaccines at the mucosa. For construction of candidate vaccines, it is important to define epitopes evoking antibodies capable of cross-neutralizing different HIV-1 viral strains. Monoclonal and polyclonal antibodies are useful reagents in this respect (D'Souza et al., 1991). In chapter 7 such variant specific and group specific neutralizing antibodies were raised using synthetic peptides. In addition, these antibodies can be used to study mechanisms involved in virus-cell fusion and syncytium formation. Finally, they are of use to probe the role of selected amino acid sequences in determining cell tropism, growth characteristics and syncytium inducing capacity.

CHAPTER 4

FOLLICULAR TRAPPING OF IMMUNE COMPLEXES

PRODUCTION OF A MONOMERIC ANTIGEN-ENZYME CONJUGATE TO STUDY REQUIREMENTS FOR FOLLICULAR IMMUNE COMPLEX TRAPPING

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ABSTRACT

Studies concerning the localization of immune complexes in lymphoid follicles and the involvement of these trapped immune complexes in the regulation of the immune response have thus far been performed with poorly defined complexes in terms of size and composition. For that reason, the minimum requirements for trapping in terms of number of antigen- and antibody molecules present in immune complexes could not be determined.

We here describe the production and *in vivo* use of a monomeric HSA-HRP antigen-enzyme conjugate, readily demonstrable in cryostat sections and ELISA. This conjugate was obtained by combining the glutaraldehyde coupling-method with chromatography to fractionate monomeric and multimeric constituents. SDS-PAGE analysis showed that the conjugate consisted of a single molecular species of 109 kD, whereas the often used periodate oxidation coupling method yielded a heterogeneous population of multimeric, oligomeric and monomeric molecules.

We investigated the minimal size requirements for the composition of immune complexes to be trapped in murine spleen follicles using three different conjugates (monomeric HSA-HRP, multimeric HSA-HRP and multimeric HSA-HRP-Penicillin) and a panel of anti-HSA and anti-Penicillin monoclonal antibodies. We demonstrate that the smallest immune complexes, consisting of one antibody and two conjugate molecules, do not localize in splenic follicles. Immune complexes prepared with a single monoclonal antibody localize in follicles only if the epitope recognized occurs repeatedly on the antigen.

The relevance of these results for physiological follicular trapping of protein antigens is discussed. The described method for the production of monomeric enzyme-labeled protein applicable in histochemistry and ELISA should prove useful to prepare other conjugates of defined size for studies of trapping and other applications.

INTRODUCTION

A small fraction of antigen-antibody immune complexes localizes in lymphoid follicles of lymph nodes (Humphrey and Frank, 1967) and spleen (Van Rooijen, 1972) directly after formation during the humoral immune response or after passive administration of immune complexes pre-formed *in vitro*. These trapped immune complexes remain present on the surface of follicular dendritic cells (FDC) for prolonged periods (Mandel et al., 1980; Tew et al., 1980). Follicular trapping requires the presence of specific antibody with an intact Fc-portion (Herd and Ada, 1969) and the presence of complement (Papamichail et al., 1975). Mouse immunoglobulin isotypes can be ranked in the order IgG2, IgG1 and IgA of decreasing trapping

ability whereas IgM does not seem to mediate trapping (Klaus, 1979; Phipps et al., 1980). This parallels the ability of the different isotypes to activate complement, with the exception of IgM.

Several functions have been ascribed to these persisting immune complexes: they could be involved in the antigen-specific feedback regulation of the humoral immune response by the exposure and coverage of antigenic determinants (Tew et al., 1980). This mechanism could be responsible for the cyclical production of antibody as seen in several experimental systems (Weigle, 1975). Klaus showed that immune complexes evoke anti-idiotypic responses, compatible with network theories of immunoregulation. He also demonstrated that immune complexes formed in antigen-excess are far more effective in generating B memory cells than antigen alone (review by Klaus, 1982 and Klaus et al., 1980). On the other hand, immune complexes formed in antibody excess may inhibit the generation of memory B-cells in the follicles (Van Rooijen, 1980). In two recent elegant studies (Szakal et al., 1988; Kosco et al., 1988) immune complexes-coated beads, termed iccosomes, were reported to detach from the dendrites of the FDC. These iccosomes are subsequently endocytosed by tingible body macrophages and germinal center B-cells. These cells are capable of *in vitro* presentation of *in vivo* obtained antigen to T-cells.

In addition to these physiological functions of follicular immune complexes, we have hypothesized that they may be involved in human immunodeficiency virus (HIV-1) induced histopathology (Laman et al., 1989). Mechanism of follicular trapping of different types of antigens (thymus dependent and thymus independent), and the role of follicular immune complexes have been discussed in more detail by Laman et al. (in press, a).

Thus, a body of evidence exists that immune complexes trapped in lymphoid follicles are involved in the regulation of the immune response and the generation of immunological memory. This warrants further investigations into the mechanisms underlying trapping and interactions of immune complexes with cells present in the follicle. Until now, studies concerning trapping have been performed with poorly defined complexes in terms of size and antigenic structure. It is important to define minimal size/composition requirements of immune complexes with respect to questions which such as which immune complexes will trap and which will not and if epitopes in immune complexes are exposed or covered, influencing the immune response. Therefore, the aim of the present study was to develop an antigen-enzyme conjugate of defined size for use in enzymehistochemical studies of immune complex trapping.

A monomeric HSA-HRP conjugate was produced and used to investigate the minimal size requirements of immune complexes to be trapped. We determined if small, trimolecular immune complexes, constructed from one anti-HSA antibody and

two conjugate molecules, localize in murine splenic follicles. In addition, using a multimeric HSA-HRP-Penicillin (Pen) conjugate and anti-Pen monoclonal antibodies (MAb), we determined if an antibody of a single specificity is sufficient to mediate trapping if its epitope occurs repeatedly on the antigen.

MATERIALS AND METHODS

ANIMALS

Female B6D2(F1)-mice (Bomholtgard, Rye, Denmark), aged 8-16 weeks, were kept in macrolon cages under a 11 h dark/13 h light regime at 20 oC with free access to acidified water (pH 3.0) and pelleted mouse food (Hope Farms, Woerden, The Netherlands).

ANTIBODIES AND CHEMICALS

Polyclonal rabbit anti-HSA hyperimmune serum was obtained from the Netherlands Red Cross Blood Transfusion Service (Amsterdam, The Netherlands) and mouse anti-HSA MAb SA (IgG1) from Sanbio (Uden, The Netherlands). Mouse anti-HSA antibodies 1,2 and 3 were a kind gift of Prof.dr. G.J. Fleuren (University of Leiden, The Netherlands). The anti-Pen MAb 4,7 and 9 (IgG), which recognize different sites of the hapten Penicillin (Pen), have been described by de Haan et al. (1979; 1985). Diaminobenzidinetetrahydrochloride (DAB) was obtained from Sigma Chem. Co. (St. Louis, MO), horseradish peroxidase (HRP; RZ 3.0) from Boehringer (Mannheim, FRG) and human serum albumin (HSA) from Miles (UK). Other chemicals were purchased from Merck (Darmstadt, FRG).

PRODUCTION OF MONOMERIC HSA-HRP

HSA (25 mg in 2 ml 0.15 M NaCl) was dialysed 18 h against 0.15 M NaCl. Monomeric HSA was fractionated from trimeric and dimeric HSA by column chromatography on Ultrogel AcA-44. HSA was coupled to HRP by the two-step glutaraldehyde method according to Avrameas and Ternynck (1971) as modified by and Streefkerk (1979). 10 mg HRP was dissolved in 0.2 ml 0.1 M phosphate buffer (pH 6.8), containing 1.25% glutaraldehyde. After 18 h at room temperature the solution was chromatographed on Ultrogel AcA-44 to remove excess glutaraldehyde and oligomeric HRP (Boorsma and Streefkerk, 1976). The fractions containing the monomeric activated HRP were pooled and concentrated to 10 mg/ml and the monomeric HSA (5 mg in 1 ml 0.15 M NaCl) was added. The pH was raised to 9.0-9.5 with 0.1 M sodium carbonate-bicarbonate buffer (pH 9.5). After 24 h at 4 oC, 0.1

ml of 0.2 M lysine-HCl solution was added. To separate monomeric HSA-HRP from uncoupled HSA and HRP, the reaction-mixture was filtrated on Ultrogel AcA-44 once more and the appropriate fractions were pooled, concentrated and stored at -20 oC until use. Using similar methods, we have previously produced multimeric TNP-poly-L-lysine-HRP conjugates (Claassen and Van Rooijen, 1985).

PRODUCTION OF MULTIMERIC HSA-HRP AND HSA-HRP-PEN

HSA-HRP was prepared according to the periodate method (Avrameas and Ternynck, 1971; Wilson and Nakane, 1978). 4 mg HRP was dissolved in 1 ml distilled water. After addition of 0.2 ml of a fresh 0.1 M NaIO4 solution the mixture was stirred for 20 min (colour turned from brown to green). The solution was dialysed against 0.01 M sodium acetate buffer pH 4.4 at 4 °C for 20 hr. The pH was raised to 9.5 by adition of 20 microliter 0.2 M sodium carbonate-bicarbonate buffer (pH 9.5) and immediately 4 mg HSA was added in 1 ml 0.01 M sodium carbonate-bicarbonate buffer (pH 9.5). After gentle mixing, the reaction was allowed to proceed for 2 hr at room temperature. 0.1 ml of a freshly prepared sodium borohydride solution (4 mg/ml distilled water) was added and the mixture was left at 4 oC for 2 hr. Hereafter the solution was dialysed against PBS and stored at - 20 °C.

HSA-HRP-Pen was prepared by adding 30 mg Pen to 10 mg multimeric HSA-HRP in 2 ml PBS. The pH was raised to 10 by the addition of 0.1 M NaOH. The reaction was allowed to proceed for 8 h. Thereafter the reaction mixture was dialysed against PBS. The number of Penicilloyl groups introduced was determined by the penamaldate method (Parker et al., 1962).

ANALYSIS OF CONJUGATES AND IMMUNE COMPLEXES

Molecular weight and purity of the different conjugates was assessed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) by running 40 microgram samples on 1 mm thick slab gels (7-17%) essentially as described by Laemmli (1970). Size of immune complexes and their constituents was determined by running samples in the same concentrations as used in the *in vivo* experiments over a Superose-6 column (Pharmacia; range 5-5.000 kD; 10 mm x 30 cm). PBS was used as elution buffer at a flow rate of 0.3 ml/min. The eluent was monitored at 280 nm with a UV-1/214 ultraviolet monitor (Pharmacia). The column was calibrated with markers of known molecular weight such as IgG (150 kD), HSA (69 kD), and HRP (40 kD).

PREPARATION, ADMINISTRATION AND DETECTION OF IMMUNE COMPLEXES

60 microgram of the conjugate was thoroughly mixed with a two-fold molar excess of the antibody in PBS in a total volume of 200 microliter per mouse. This mixture was incubated for 1 h at 37 oC to allow complex formation and injected intravenously immediately hereafter. Mice were sacrificed 24 h after injection of immune complexes by cervical dislocation and spleens were snap-frozen in liquid nitrogen. 8 micrometer sections were fixed in acetone for 10 min and air-dried. HRP-activity was demonstrated by incubation with AEC (3-amino-9-ethylcarbazole) for 10 min. 4 mg AEC was dissolved in 250 microliter N,N-dimethylformamide, and added to 9.75 ml sodium acetate buffer (0.05 M, pH 5). Just before use, 50 microliter 3% hydrogenperoxide was added. The reaction was stopped by transferring the slides to PBS. Slides were mounted with glycerol/gelatin.

RESULTS

To obtain a monomeric antigen-enzyme conjugate, monomeric HSA was mixed with monomeric glutaraldehyde activated HRP. After the coupling reaction the mixture was chromatographed to dispose of uncoupled HSA and HRP. Figure 1 shows the elution pattern of this last purification step. Protein (HSA and HRP) was detected at 280 nm and HRP at 403 nm. Peak I represents the monomeric conjugate: appropriate fractions were pooled and concentrated. Peak II represents the non-conjugated HSA; peak III represents non-conjugated HRP. From the elution volume a MW of 109 kD was determined for the conjugate. Figure 1 shows that the efficiency of the glutaraldehyde method is rather low: the conjugate in peak I contains only a small amount of the total protein.

Monomeric HSA-HRP conjugates and the multimeric HSA-HRP-Pen conjugates were analysed by SDS-PAGE (figure 2). A mixture of components of known molecular weight, including HSA (69 kD) and alkaline phosphatase (100 kD; Claassen et al., 1986) was used for reference. The electrophoresis pattern shows the differences in MW and homogeneity of the conjugates prepared by the two different coupling procedures: periodate treatment of HRP yields a heterogenous population of multimeric molecules of different MW. Some of these molecules are too large to even enter the running gel and they accumulate in the stacking gel (stacking gel not shown). The modified glutaraldehyde coupling method gives rise to a pure monomeric HSA-HRP conjugate with a MW of 109 kD. In addition to the band of the conjugate, a 69 kD band of HSA and two bands of approximately 40 kD which represent the two different isoenzymes of HRP in the commercial preparation can be seen.

After preparation of the HSA-HRP-Pen conjugate, the penamaldate method

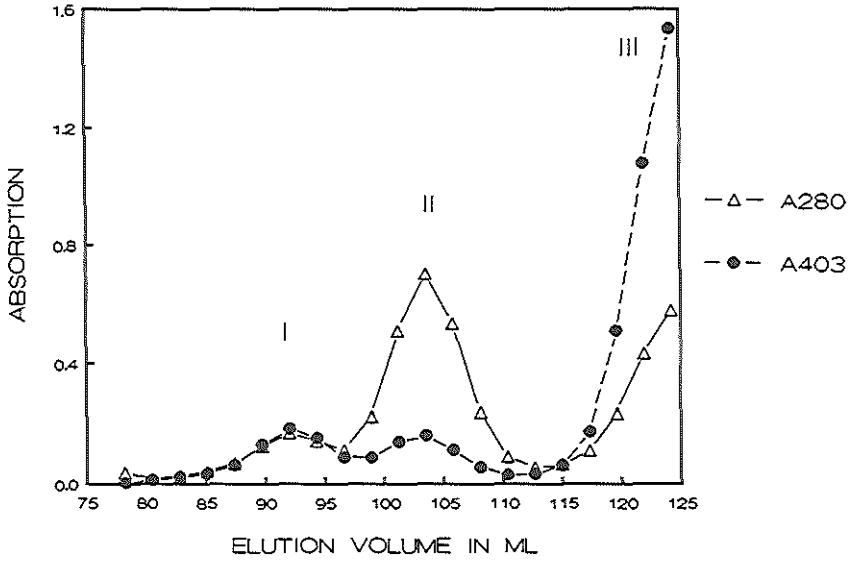


FIGURE 1. ACA-44 COLUMN CHROMATOGRAPHY OF THE HSA-HRP CONJUGATION-MIXTURE

The reaction mixture of monomeric HSA and monomeric glutaraldehyde-activated HRP was applied to a 60 x 2 cm AcA-44 column, equilibrated with PBS pH 7.0, and eluted with the same buffer at a flow rate of 3.7 ml/cm2/hr. 2.3 ml fractions were collected. Fractions were analysed at 280 nm (protein optimum) and 403 nm (HRP optimum). Peaks, indicated as I, II and III, represent the monomeric HSA-HRP, non-conjugated HSA and non-conjugated HRP, respectively. See Results section for further discussion.

revealed that an average of 7 Penicilloyl groups was present per 100 kD HSA-HRP.

The MW of the different antibodies, conjugates and immune complexes was determined by gelchromatography on a Superose-6 size exclusion column (range 5 - 5,000 kD). Results (summarized in Table 1) again demonstrate the difference in size of conjugates, depending on the coupling method used. The MW of the immune complexes also demonstrates the difference in size between immune complexes made with polyclonal or monoclonal antibodies: anti-HSA MAb are able to link only two molecules of the monomeric conjugate whereas polyclonal anti-HSA gives rise to immune complexes of high MW by extensive crosslinking.

After intravenous administration of immune complexes of different size and composition, murine spleen sections were treated enzymehistochemically to reveal

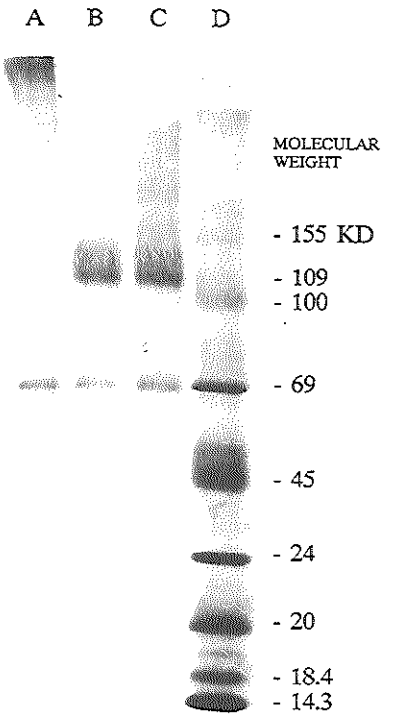


FIGURE 2. SDS-PAGE ANALYSIS OF HSA-HRP CONJUGATES

Lane A: multimeric HSA-HRP, prepared by the periodate oxidation method. Lane B and lane C: two monomeric HSA-HRP conjugates. Lane D: low molecular weight markers. Molecular weight in kD is indicated at the right. A fraction of the multimeric HSA-HRP (Lane C) was too large to enter the running gel and remained in the stacking gel (not shown).

TABLE 1. MOLECULAR WEIGHT OF IMMUNE COMPLEXES AND THEIR CONSTITUENTS AS DETERMINED BY SIZE EXCLUSION CHROMATOGRAPHY

SAMPLE	MOLECULAR WEIGHT IN kD
HSA	69
HRP	40
HSA-HRP (monomeric)	109
HSA-HRP (multimeric)	109 - >500
HSA-HRP-PEN	109 - >500
anti-HSA MAb (1, 2, 3 and SA)	150
anti-HSA hyperimmune serum (HY)	150
HSA-HRP (monomeric) + HY	>500
HSA-HRP (monomeric) + anti-HSA 1,2,3 and SA	370
HSA + HY	>500
HSA + anti-HSA 1,2,3 and SA	>500

HRP-activity. Results are shown in Table 2. The different conjugates were all readily detectable in histological sections when complexed with HY. Monomeric HSA-HRP did not localize when complexed with one or more of the anti-HSA MAb. This result was confirmed with several concentrations of the different MAb (data not shown). Multimeric HSA-HRP localized when combinations of anti-HSA MAb are used, but was only trapped if anti-HSA 1 is used as single complexing antibody. Figure 3 shows the histological pattern of follicular trapping of HSA-HRP. As a reference, trapping of peroxidase-anti-peroxidase is shown.

HSA-HRP-Pen was found in splenic follicles after complexing with either anti-Pen 4 or 7 but not after complexing with anti-Pen 9. Interestingly, anti-Pen 9 does not mediate trapping and this antibody blocks the trapping process mediated by anti-Pen 7, even if the conjugate is incubated with anti-Pen 7 for 30 min prior to addition of anti-Pen 9. Trapping through anti-Pen 4 is not influenced by the addition of 9.

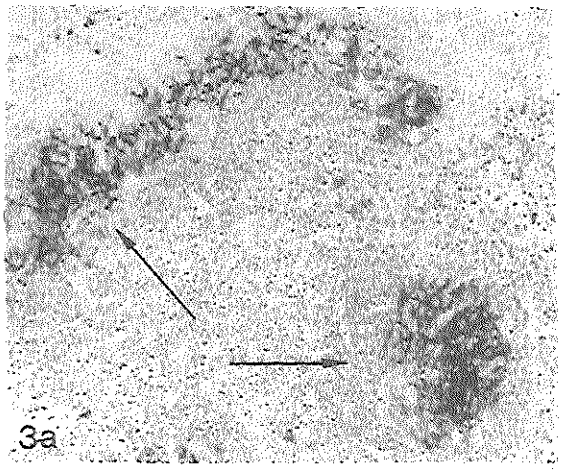
TABLE 2. ENZYMEHISTOCHEMICAL DEMONSTRATION OF IMMUNE COMPLEXES IN MURINE SPLENIC FOLLICLES

COMPLEXES INJECTED	HRP-ACTIVITY IN SPLENIC FOLLICLES
HSA-HRP (multimeric) + HY	+
HSA-HRP (monomeric) + HY	+
HSA-HRP (monomeric) + anti-HSA 1 + 2 + 3 + SA	+
HSA-HRP (multimeric) + all combinations of 2 anti-HSA MAb	+
HSA-HRP (multimeric) + anti-HSA 1	+
HSA-HRP (multimeric) + anti-HSA 2	-
HSA-HRP (multimeric) + anti-HSA 3	-
HSA-HRP (multimeric) + anti-HSA SA	-
HSA-HRP-Pen + HY	+
HSA-HRP-Pen + anti-Pen 4	+
HSA-HRP-Pen + anti-Pen 7	+
HSA-HRP-Pen + anti-Pen 9	-
HSA-HRP-Pen + anti-Pen 9 + 4	+
HSA-HRP-Pen + anti-Pen 9 + 7	-
HSA-HRP-Pen + anti-Pen 9 + 7 + 4	+

* Mice received an i.v. injection of the complexes dissolved in PBS. Four mice were tested per combination.

FIGURE 3. LOCALIZATION OF IMMUNE COMPLEXES IN SPLENIC FOLLICLES

Mice received an intravenous injection of immune complexes. Cryostat sections of the spleen were stained with AEC to reveal HRP-activity, resulting in bright red staining. Red-stained immune complexes form a lattice between the cells of the follicle. Figure 3a. Localization of commercial peroxidase-anti-peroxidase immune complexes (PAP; Dakopatts; 200 μl undiluted, i.v.), to show normal follicular localization of immune complexes (arrows). Counterstained with haematoxylin. x 100. Figure 3b. Localization of monomeric HSA-HRP complexed with anti-HSA hyperimmune serum (HY) (arrow). Not counterstained. x 200.



DISCUSSION

With the aim to study the minimal size requirements for follicular trapping of immune complexes, we constructed a pure monomeric HSA-HRP antigen-enzyme conjugate by a modification of the glutaraldehyde coupling method of Avrameas and Ternynck (1971). The monomeric conjugate reacted well in histological sections after *in vivo* administration and in an ELISA-system.

Commercially obtained HRP and HSA preparations both contain monomers and small amounts of dimers and trimers. To prepare a monomeric conjugate it is therefore necessary to first purify the monomeric fractions of both proteins before coupling. HRP was chromatographed after glutaraldehyde activation and the monomeric fraction was added to chromatographed, monomeric HSA. To separate

the conjugate from free HSA and HRP, the reaction-mixture was again chromatographed. SDS-PAGE analysis showed that a pure monomeric fraction with a MW of 109 kD was obtained. This MW was in accordance with the data from size exclusion chromatography. In contrast, the periodate coupling method gave rise to a heterogenous population of monomeric to large polymeric molecules. The uncoupled HSA and HRP found in SDS-PAGE analysis may result from dissociation of the conjugate during sample preparation (including boiling in SDS). Alternatively, pseudo-conjugates (Deen et al., 1990) of HSA aspecifically sticking to HRP may have formed, which do not dissociate during column chromatography.

A drawback of the glutaraldehyde coupling-method is the rather low yield of conjugate: only a small fraction of both proteins is coupled. The surplus of activated HRP cannot be used again but the antigen, which is usually more precious, can be recycled, if necessary.

The coupling of HRP not only adds 40 kD to the 69 kD moiety of HSA, but may also affect the accessibility of antibody epitopes on the HSA-molecule. Our data obtained with size-exclusion chromatography show that the monomeric HSA-HRP conjugate can only form small trimolecular immune complexes with a mixture of anti-HSA MAb whereas normal HSA also gives rise to larger immune complexes. Apparently, HRP together with the MAb that binds first obscures the binding site for the second antibody. This did not hamper the *in vivo* studies, however, as the anti-HSA hyperimmune serum was able to generate large complexes.

HRP contains only few lysine residues with free epsilon amino-groups which can be activated by glutaraldehyde but globular proteins have many of these accessible amino groups (Boorsma, 1983). For that reason, the final monomeric conjugate can contain many sterically different molecules with HRP attached in several orientations to distinct sites on the protein, interfering with antibody binding.

The size-defined HSA-HRP conjugate enabled us to prepare immune complexes of predefined size and composition, with which we sought to define the minimal size requirements for immune complexes to localize in lymphoid follicles. The minimal size of immune complexes is important when one considers that the antibody-responses to different epitopes on complex protein antigen may not evolve simultaneously. It is likely that the most immunogenic epitope will also be the first to evoke a humoral response. Are these first-appearing antibodies capable of mediating follicular trapping when this epitope is expressed only once on the antigen and crosslinking of the antigen to form large immune complexes will thus be impossible? To address this question we prepared small immune complexes, consisting of one antibody and two conjugate molecules and immune complexes formed with one MAb against one type of repeated epitope. These immune complexes have only one Fc-portion and are thus inable to activate complement. Our data show that whereas immune complexes of high MW, formed with polyclonal

anti-HSA and monomeric HSA-HRP, do localize, the trimolecular complex does not. This indicates that, if the antibody response against complex antigens is indeed staged in reactions to epitopes of different immunogenicity, small immune complexes formed by the first-appearing antibody against a non-repeating epitope will not localize in the follicles of lymphoid organs. Taken together with the fact that IgM, the first-appearing immunoglobulin isotype in the primary response, is not able to mediate trapping, these data argue against a role of follicularly trapped immune complexes in early events of the immune response.

With the HSA-HRP-Pen conjugate and the anti-Pen MAb we demonstrated that antibodies of a single specificity can mediate trapping if the complementary epitope occurs repeatedly on the antigen. This indicates that an *in vivo* response against only one type of repeated epitope will be sufficient to evoke trapping. An interesting observation was the fact that anti-Pen 9, contrary to 4 an 7, is not able to mediate trapping and even blocks the effect of 7. We speculate that this reflects a process of sterical hindrance that abrogates the complement-activating function of the Fc-portion or blocks the Fc-receptor binding domains on the Fc-moiety.

Our conclusions are in agreement with data described by Embling et al. (1978): these authors studied trapping requirements not with immune complexes proper but with preparations of aggregated human IgG. They found that trimerization of IgG is a minimal requirement for trapping to occur. Their and our results confirm the notion that the ability of immune complexes to fix complement is crucial to their subsequent follicular localization (Papamichail et al., 1975). This complement-dependence is generally explained by the transport of immune complexes from the marginal zone to the follicular center by B-cells carrying Fc-receptors and C3-receptors (Brown et al., 1971; Klaus and Humphrey, 1986). Only complexes that contain C3-fragments and Fc-portions would be crosslinked on the surface of marginal zone B-cells strongly enough to be transported into the follicle. The inability of IgM to mediate trapping would be related to the lack of IgM Fc-receptors on B-cells. This explanation may not be sufficient however, because experimental evidence indicates that immune complexes may also reach the follicle without cell-mediated transport (cf. Laman et al., 1990). Maeda et al. (1988) have provided evidence that locally produced C1q may be involved in retention of immune complexes in lymphoid follicles.

In conclusion, the described coupling-method offers the possibility to construct size-defined conjugates of use in *in vivo* enzymehistochemical and in immunochemical techniques. With such conjugates the relations between composition, anatomical localization and the function of immune complexes in immunoregulation can be studied.

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The references to this article are included in the References section following chapter 8.

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Mechanism of follicular trapping: localization of immune complexes and cell remnants after elimination and repopulation of different spleen cell populations

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SUMMARY

The role of marginal zone macrophages, marginal metallophilic macrophages (marginal metallophils) and marginal zone lymphocytes in the follicular trapping of immune complexes was investigated in a detailed elimination and repopulation study. Intravenous injection of liposome-encapsulated dichloromethylene diphosphonate (Cl_2MDP) resulted in a complete and lasting elimination of marginal zone macrophages and marginal metallophils, while the number of marginal zone B-lymphocytes was temporarily reduced. By means of image analysis of light-microscopic images we quantified the repopulation of the above cell types and the presence of immune complexes during the repopulation process. Trapping of peroxidase-anti-peroxidase complexes was reduced up to Day 3 after administration of Cl_2MDP-liposomes, but reached control values on Day 5, before reappearance of the different cell types. Therefore, marginal zone macrophages and marginal metallophils are neither directly nor indirectly involved in the transport of immune complexes to splenic follicles. It is unlikely that marginal zone B cells play a role in the transport of complexes, as a substantial reduction in B-cell number did not impair follicular trapping. At different time-points after treatment with Cl_2MDP-liposome treatment, three macrophage markers (acid phosphatase, ligand for ERTR-9 and ligand for MOMA-2) were found in splenic follicles of several animals, but not in control animals. The presence of these macrophage markers in splenic follicles implies that soluble and particulate cell remnants migrate to the follicle and are retained there without the involvement of specific antibody and complement. Collectively, the data showing trapping of immune complexes despite the absence of several candidate transporter cell types and the localization of cellular remnants to splenic follicles provide evidence against a cell-mediated transport of immune complexes. The data argue in favour of diffusion as a transport mechanism of both immune and non-immune compounds to the follicle.

INTRODUCTION

Small quantities of immune complexes localize in the follicles of lymph nodes (Humphrey & Frank, 1967) and spleen (Van Rooijen, 1972) shortly after injection of preformed complexes, or as soon as complexes are formed in the primary reponse. This process is called immune complex- or antigen-trapping. Trapped complexes remain undegraded in the follicles for prolonged periods (reviewed by Mandel *et al.*, 1980) and are believed to be involved in the generation and maintenance of immunological memory, the feedback inhibition of antibody synthesis and anti-idiotypic reactions to self-idiotopes (reviewed by Klaus *et al.*, 1980).

Abbreviations: Cl_2MDP, dichloromethylene diphosphonate (DMDP); FDC, follicular dendritic cell; HRP, horseradish peroxidase; PALS, periarteriolar lymphocyte sheath; PAP, peroxidase-anti-peroxidase; PBS, phosphate-buffered saline.

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Trapping of an antigen requires the presence of specific antibody with an intact Fc-portion (Herd & Ada, 1969) and the presence of complement (Papamichail *et al.*, 1975), as complexes are bound by C3b-receptors and Fc-receptors on the surface of the extensions of the follicular dendritic cells (FDC) (Nossal *et al.*, 1968; Hanna & Szakal, 1968).

Opinions differ as to the mechanism by which immune complexes migrate from the marginal zone to the follicle centre. This migration could be either passive by diffusion or active through complex-carrying cells. Several authors have postulated active transport by lymphocytes (Veerman & Van Rooijen, 1975; Brown *et al.*, 1971; Gray *et al.*, 1984; Heinen *et al.*, 1986; Braun *et al.*, 1987). Macrophages (White, French & Stark, 1970) and migrating FDC-precursors (Szakal, Holmes & Tew, 1983) have also been suggested as complex-transporting cells.

Passive transport to the follicles has been demonstrated with peroxidase-anti-peroxidase (PAP) complexes (Kamperdijk, Dijkstra & Döpp, 1987) and with immunologically inert

particles (Chen, Adams & Steinman, 1978; Groeneveld, Eikelenboom & Van Rooijen, 1983).

Recently, we developed a model in which splenic macrophages are eliminated with liposome-encapsulated dichloromethylene diphosphonate (Cl_2MDP) (Van Rooijen & Van Nieuwmegen, 1984; Van Rooijen & Claassen, 1988). One day after intravenous administration of drug-filled liposomes, marginal zone macrophages, marginal metallophilic macrophages (marginal metallophils) and red pulp macrophages disappear. Marginal zone B cells are reduced in number, but are not completely eliminated (Claassen, Kors & Van Rooijen, 1987; Van Rooijen, Van Nieuwmegen & Kamperdijk, 1985). This model allows studies on the function of the different cell types as they have differential repopulation kinetics.

The aim of the present study, therefore, was to determine if marginal zone macrophages, marginal metallophils and marginal zone lymphocytes (T and B cells) are involved in the trapping of immune complexes in murine splenic follicles, either by active transport or by maintaining the microenvironment necessary for the migration of complex-transporting cells. To this end, we investigated the correlation between the presence of the different cell types in relation to follicular trapping of PAP complexes.

MATERIALS AND METHODS

Experimental design

Mice received an intravenous injection of 200 μl of a Cl_2MDP-liposome suspension containing 2 mg Cl_2MDP on Day 0. On Day -1, 1, 2, 4, 8, 12, 16 and 32, a second injection with 200 μl PAP was given. Each group consisted of four mice. Controls consisted of mice given 200 μl phosphate-buffered saline (PBS) or 200 μl PBS-containing liposomes. Twenty-four hours after administration of PAP, the spleen was taken and frozen in liquid nitrogen for histo- and immunochemistry. The experiment was performed twice: once with B6D2 and once with BALB/c mice.

Animals

Male (B6D2)F_1 mice (Bomholtgård, Rye, Denmark) or BALB/c mice (TNO Central Animal Facility, Rijswijk, The Netherlands), aged 8–16 weeks, were kept in macrolon cages under a 11 hr dark/13 hr light regime at 20° and were given acidified water (pH 3) and pelleted mouse food (Hope Farms, Woerden, The Netherlands) *ad libitum*.

Chemicals and reagents

3-Amino-9-ethylcarbazole, cholesterol, naphtol AS-BI-phosphate and 3,3-diaminobenzidine were obtained from Sigma Chemical Co. (St Louis, MO). Cl_2MDP was a kind gift from Procter and Gamble (Cincinnati, OH). PAP (rabbit), rabbit anti-rat Ig-horseradish peroxidase (HRP) and rabbit anti-mouse IgM-HRP, were obtained from Dako (Copenhagen, Denmark) and Cappel Lab. (Cochranville, PA), respectively. Monoclonal antibodies (mAb) MOMA-1, used to demonstrate marginal metallophils (Kraal & Janse, 1986), and MOMA-2, used as a pan-macrophage marker (Kraal, Rep & Janse, 1987), were a kind gift from Dr G. Kraal, Vrije Universiteit. ERTR-9, used to demonstrate marginal zone macrophages (Dijkstra *et al.*, 1985; Van Vliet, Melis & Van Ewijk, 1985), was a kind gift from Dr C. D. Dijkstra, Vrije Universiteit. Thy-1 a kind gift from Dr Kraal (Ledbetter & Herzenberg, 1979) was used to detect T cells.

Preparation of Cl_2MDP-liposomes

Multilamellar liposomes were prepared as described earlier (Van Rooijen & Van Nieuwmegen, 1984). To summarize the procedure briefly, 86 mg phosphatidylcholine and 8 mg cholesterol were dissolved in chloroform in a round-bottomed flask. By vacuum rotary evaporation at 37 °, a thin film was formed on the wall of the flask. Cl_2MDP (1·89 g dissolved in 10 ml PBS) was enclosed by gentle shaking for 10 min. The suspension was kept for 2 hr at room temperature and sonicated for 3 min at 20 °C at 50 Hz. After an additional 2 hr at room temperature free Cl_2MDP was removed by centrifugation (100,000 *g*, 30 min). Liposomes were resuspended in 4 ml PBS. The amount of liposome-encapsulated Cl_2MDP was determined according to Claassen & Van Rooijen (1986).

Histochemistry and immunocytochemistry

Cryostat sections (8 μm) of spleen tissue were fixed in acetone containing 0·02% H_2O_2 to minimize endogenous peroxidase activity for 10 min (Streefkerk, 1972). In order to detect PAP complexes, sections were fixed without H_2O_2 and stained after air-drying with 3-amino-9-ethylcarbazole (AEC) for 10 min (0·4 mg/ml in 0·05 M acetate buffer, pH 5·0; AEC was first dissolved in 62·5 μl/mg NN-dimethylformamide). The reaction was stopped by transferring the slides to PBS. Acid phosphatase activity was demonstrated by incubation with naphtol AS-BI phosphate and pararosaniline for 30–45 min at 37 °C. Monoclonal antibody staining was performed by incubating sections, after air-drying and fixing in acetone/H_2O_2, for 1 hr at 4 °C, with the appropriate dilution of the mAb supernatant, rinsed three times in PBS and incubated with the appropriate conjugate and normal mouse serum for 1 hr at room temperature. After rinsing three times in PBS, HRP activity was demonstrated by incubation for 10 min with 3,3-diaminobenzidine (0·5 mg/ml in 0·05 M Tris-HCl, pH 7·6, containing 0·01% H_2O_2). The reaction was stopped by transferring the slides to PBS. Slides (either counterstained or not with haematoxylin for 15 seconds) were mounted in glycerin–gelatin or in malinol after dehydration.

Quantification of cell markers

Histochemical results were quantified on an Artek counter (model 880; Farmingdale, NY) coupled to an external videomicroscope. The total stained surface was taken for image analysis, as described before (Claassen *et al.*, 1988). Results were scored on coded samples (blind) with automated data gathering. Data were analysed with the two-sample Student's *t*-test for comparison of two empirical means in a normally distributed population (Sachs, 1984). Statistical significance was assumed when $P < 0{\cdot}05$.

RESULTS

Trapping of immune complexes after administration of Cl_2MDP-liposomes

Trapping of immune complexes at any given time after treatment with Cl_2MDP-liposomes was indistinguishable from the histological pattern seen in untreated controls. Figure 1a clearly shows this for PAP complexes injected 8 days after administration of Cl_2MDP-liposomes. However, a decrease in the detectable amount of immune complexes was observed when PAP was given 1 (Fig. 1c) or 2 days after Cl_2MDP-liposomes (Fig. 2). A

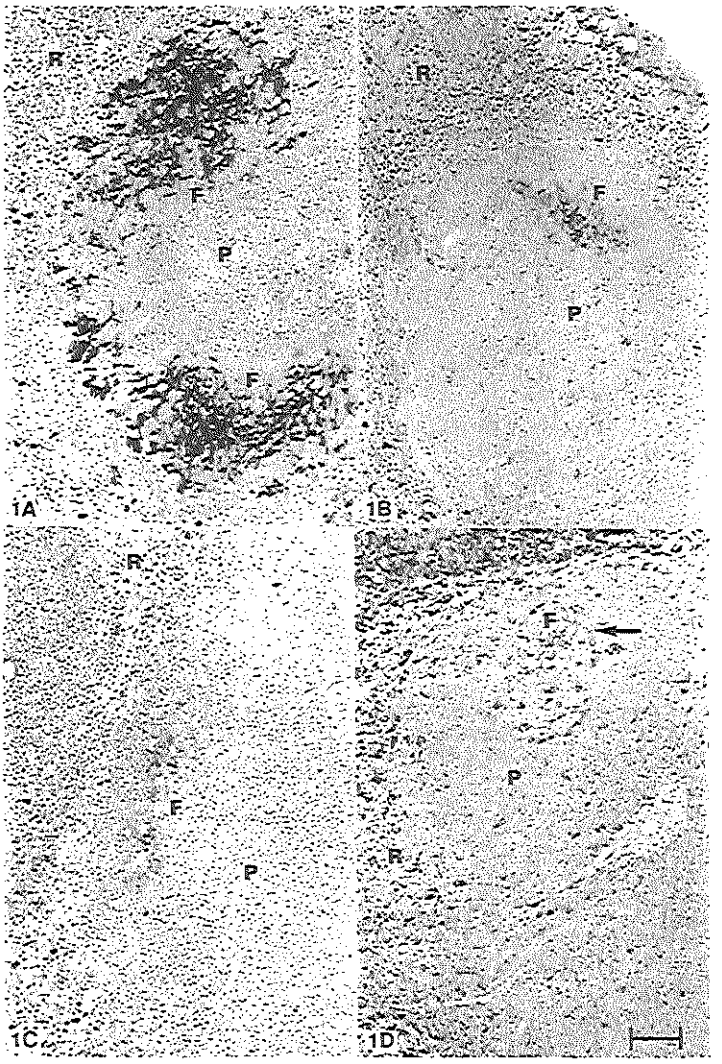

Figure 1. Section of a splenic follicle of a mouse which received PAP (a) 8 days after, (b) 1 day before, or (c) 1 day after Cl_2MDP-liposomes. The staining in (a) is comparable with staining observed in mice that received no liposomes. (d) Shows a section of a splenic follicle of a mouse that received Cl_2MDP-liposomes but no PAP, demonstrating MOMA-2 activity. The mouse was killed 16 days after administration of the liposomes. Sections were counterstained with haematoxylin. Magnification × 75. Bar represents 100 μm. R, red pulp; F, follicle; P, periarteriolar lymphocyte sheath.

similar decrease was also observed when PAP was given 1 day before Cl_2MDP-liposomes (Fig. 1b). This decrease was of only very short duration because PAP complexes given 4 days after Cl_2MDP-liposomes were found in quantities identical to controls. No further decrease, or increase, was observed when complexes were administered at later time-points after Cl_2MDP-liposomes (Fig. 2).

After administration of Cl_2MDP-liposomes, and subsequent elimination of macrophages from the spleen, macrophage proteins (markers) were found in the splenic follicles of several animals in a dendritic pattern resembling immune complex trapping. Acid phosphatase (characteristic for all macrophages), ERTR-9-ligand (marginal zone macrophages), MOMA-1-ligand (marginal metallophils) and MOMA-2-ligand (pan-macrophage marker) were found dispersed between the cells comprising the splenic follicle. Membrane markers reacting with ERTR-9 and MOMA-2 were demonstrable in the follicles up to Day 32 (and up to Day 65; results not shown), while acid

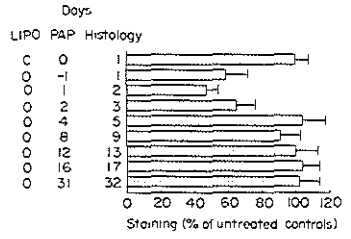

Figure 2. Image analysis data of staining for PAP complexes trapped in splenic follicles. Mice were killed at 0, 1, 2, 3, 5, 9, 13, 17 and 32 days. Cl_2MDP-liposomes (LIPO) were injected at Day 0 and immune complexes 1 day before killing, as indicated in the columns. Data are expressed as the mean of two experiments and a total of eight animals (see the Materials and Methods). C, control.

phosphatase activity decreased after Day 3. Figure 1d demonstrates MOMA-2 activity in a splenic follicle, in a web-like structure similar to the pattern characteristic for trapping.

Elimination of macrophages and reduction of marginal zone B cells in the spleen

After intravenous injection of Cl_2MDP-liposomes, a rapid decline in splenic macrophages was observed, identical to experiments described before (Van Rooijen & Claassen, 1988; Van Rooijen, 1989). In the present study we precisely quantified the characteristic markers of macrophages, T cells and B cells to obtain quantitative data in addition to already available qualitative histological findings. The enzyme-histochemical data of acid phosphatase staining show that all macrophages were already eliminated from the red pulp or marginal zone 2 days after administration of Cl_2MDP-liposomes. This finding was confirmed when the two marginal zone subpopulations of macrophages were detected by means of specific antibodies and immunohistochemistry (Fig. 3b), instead of by acid phosphatase histochemistry.

No effect of Cl_2MDP-liposomes was observed on the (relatively low) number of white pulp (follicles, inner periarteriolar lymphocyte sheath) macrophages (Fig. 3a) or T cells (Fig. 3c). A two-fold reduction in the number of B cells in the marginal zone (MZ) was observed around 2 days after treatment (Fig. 3c).

Repopulation of eliminated splenic cells

A rapid recovery to, statistically significant, normal numbers of MZ B cells was already observed within 1 week after administration of Cl_2MDP-liposomes (Fig. 3c). Red pulp macrophages repopulated more slowly but reached normal numbers around 17 days after treatment. However, marginal zone macrophages and marginal metallophils were much slower in returning to their normal location and numbers in the spleen. It was not until Day 13 that marginal metallophils were again localized in the marginal zone, and only around Day 33 that a normal number and localization was restored. The first marginal zone macrophages (ERTR-9 positive) cells were visible on Day 17, but these cells did not possess characteristic marginal zone macrophage morphology nor did they localize in the marginal zone. At 32 days after treatment only few bona fide marginal zone macrophages were observed and full restoration was observed 65 days after treatment (data not shown). This slow repopulation of marginal zone macrophages was also observed in an experimental system using chronic injection of sphingomyelin containing liposomes (Claassen *et al.*, 1988).

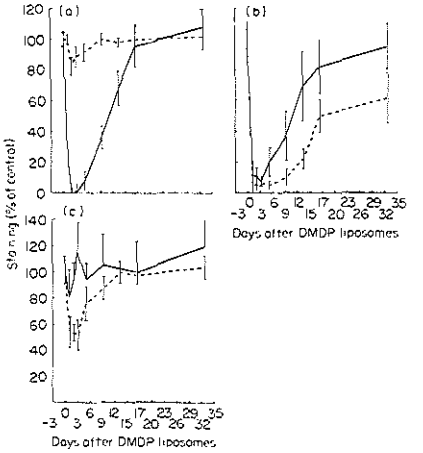

Figure. 3. Image analysis data of histochemical stainings for different murine cellular markers. (a) Acid phosphatase (pan macrophage marker); (——) red pulp and marginal zone; (----) PALS and follicles. (b) MOMA-1 (——) and ERT-9 (----) (markers for marginal metallophils and marginal zone macrophages, respectively. (c) Thy-1 (—) and IgM (---) (markers for T and B cells, respectively. Data (each point) are expressed as the mean of two experiments and a total of eight animals (see the Materials and Methods).

DISCUSSION

The present study shows that complete elimination of marginal zone macrophages and marginal metallophils and a substantial reduction in the number of marginal zone B cells does not prevent follicular trapping of PAP complexes. Furthermore, we demonstrated follicular localization and retention of macrophage determinants released from dying macrophages after elimination of these cells. We conclude that none of the investigated cell types is required for the active transport of immune complexes, but that this process occurs through passive diffusion.

Despite many publications, the mechanism of immune complex trapping and the function of trapped complexes have remained elusive. Recent reports suggesting a role of trapped complexes in restimulation of B-memory cells (Gray & Skarvall, 1988) and in the destruction of FDC in AIDS (Laman *et al.*, 1989) warrant further studies on follicular immune complexes. We here focused on the possible cell-mediated transport of immune complexes, using an *in vivo* macrophage elimination model.

Elimination and differential repopulation of macrophages in the spleen with Cl_2MDP provides a convenient model to study functional aspects of macrophage subsets. A panel of *in vitro* experiments confirmed the specificity of the method for macro-

phages. Macrophages were the only cells that were affected (eliminated) after incubation of cell suspensions from lymphoid tissues with Cl_2MDP-liposomes. No other cells were damaged or reduced in numbers and no effects on protein synthesis, cell growth, antigen presentation and (antigen-specific) T-cell proliferation were observed (Claassen, Van Rooijen & Claassen, 1990). It should be kept in mind, however, that during the first 4–5 days after *in vivo* Cl_2MDP treatment non-specific effects on other cells than macrophages can be observed. After injection, first the macrophages, and only these, take up large amounts of liposomes, which are degraded by lipases, after which the drug is released and the macrophage is killed. The dead macrophages release their lysosomal content, resulting in non-specific damage to surrounding cells (e.g. B cells; Fig. 3e). In the red pulp this damage is not as conspicuous as in the marginal zone (Van Rooijen *et al.*, 1985), since cells are replaced more rapidly in the former compartment. Consequently, the results obtained in any study with a macrophage elimination model through Cl_2MDP-liposomes should be carefully interpreted with respect to the first 4–5 days, as the number of marginal zone B cells has been reduced in addition to complete elimination of macrophages. The data in the present study obtained between Days 5 and 14 are therefore the best reflection of a spleen without macrophages.

Our present results clearly show that complete removal of marginal zone macrophages and marginal zone metallophils does not impair follicular trapping of immune complexes. These two macrophage types are therefore not involved in the active transport of these immune complexes or in the maintenance of a microenvironment necessary for the migration of other complex-transporting cells. It has been suggested that B cells are capable of transporting immune complexes by binding them to their Fc- and C3-receptors. B cells have been shown to transfer immune complexes to FDC *in vitro* (Heinen *et al.*, 1986; Braun *et al.*, 1987). It is uncertain, however, that this transfer route is also the main route *in vivo*. To date, no studies describing large numbers of migrating immune complex-bearing cells *in situ* have been published. It is also doubtful if the quantities of complexes localized in the follicles could be accounted for by the relatively small amounts that lymphocytes can transfer. In view of the present data, showing that immune complexes still localize normally when a substantial fraction of the B-cell population is gone, a role of these cells in immune complex transport is not likely. Our findings are in agreement with the data of Kamperdijk *et al.* (1987) who, on the ultrastructural level, demonstrated that PAP complexes penetrated towards the follicles of the lymph nodes in a random distribution after footpad injection, showing no preference in adherence to lymphoid or non-lymphoid cells.

The presence of various macrophage markers, released as remnants from dying macrophages, in splenic follicles shortly after treatment with Cl_2MDP-liposomes is a strong indication that non-antibody-complexed compounds may localize in splenic follicles. It is difficult to picture how and why trapping-mediating antibodies against macrophage surface markers and an ubiquitous enzyme-like acid phosphatase would be present in healthy animals. In a control experiment (results not shown) we injected purified acid phosphatase intravenously and were able to demonstrate follicular localization of this enzyme after 1 day. We therefore conclude that transport and retention of these compounds can take place without the involvement of complement and antibodies. This non-specific retention is mediated by unknown structural elements, but it may be hypothesized that mechanical retention is provided by the tight network of B cells and intertwining dendrites of the FDC. An alternative explanation for the presence of macrophage markers in follicles would be that macrophage membrane fragments carry C3b-fragments and/or immune complexes with free Fc-portions mediating trapping. This would not explain localization of acid phosphatase, however. Follicular localization of cellular non-immune compounds is in agreement with data from Cohen *et al.* (1966), Chen *et al.* (1978) and Groeneveld *et al.* (1983), who found that immunologically inert carbon particles, which are unable to evoke an immune response or to activate complement, do localize in the follicles.

We suggest that the following events take place in follicular trapping. First, immune complexes reach the marginal zone from the blood stream via the white pulp terminal capillaries. Subsequently, they diffuse to the follicle centre and are retained on the surface of FDC by Fc- and C3-receptors, which make contact with their ligands in the complex. This association is stable and may remain intact for a long time. Non-immune compounds also diffuse into the follicular web formed by the dendritic cell processes where they can be retained for a few days to several weeks. This may depend on the soluble (acid phosphatase) or particulate (membrane determinants; ERTR-9, MOMA-1 and MOMA-2) nature of the compound. This retention is purely mechanical. The present results support the model proposed by Groeneveld *et al.* (1983): for both immune complexes and non-immune compounds migration is passive by diffusion while retention is active and specific only for the former.

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CHAPTER 5

NEW METHODS TO STUDY HIV-1 SPECIFIC ANTIBODIES *IN SITU*

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Rapid Communication

Double Immunocytochemical Staining for In Vivo Detection of Epitope Specificity and Isotype of Antibody-forming Cells Against Synthetic Peptides Homologous to Human Immunodeficiency Virus-1[1]

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Many infections evoke a strong humoral immune response. Some (e.g., HIV-1, EBV, CMV) also lead to disorders of the B-cell system. Data concerning cell dysfunction are largely derived from in vitro studies, which necessarily exclude all microenvironmental influences. The aim of this study was to develop a tool for the investigation of epitope specific humoral immune responses in vivo. Mice were immunized with one of two synthetic peptides, both 21 amino acids long and homologous to regions of the HIV-1 gp160. Cryostat sections of spleen and lymph nodes were incubated with the corresponding peptide coupled to alkaline phosphatase and simultaneously incubated with peroxidase-conjugated rabbit antisera specific for mouse immunoglobulin isotypes. We were able to show simultaneous detection of epitope specificity, isotype, and localization of antibody-forming cells and immune complexes in tissue sections. It should prove useful for in vivo investigation of the development of specific (e.g., anti-HIV-1) humoral immune response, the determination of B-cell specificity in lymph node infiltrates, and the role of immune complexes in lymph node pathology. (*J Histochem Cytochem 38:457–462, 1990*)

KEY WORDS: AIDS; Alkaline phosphatase; Antibody-forming cells; Epitope; HIV-1; Immune complexes; Immunocytochemistry; Mouse; Synthetic peptides.

Introduction

Infection with the human immunodeficiency virus type 1 (HIV-1) evokes a humoral as well as a cellular immune response, but it remains obscure why this response does not lead to protective immunity (21). HIV-1 infection and subsequent development of AIDS is also accompanied by striking changes in lymphoid architecture and composition of the lymphoid tissues (22) and by several disorders of the B-cell compartment, such as polyclonal B-cell proliferation, hypergammaglobulinemia (15), and B-cell infiltrates in spleen and lymph nodes. These defects of the B-cell compartment have not been sufficiently explained as yet. As we recently discussed, immune complexes trapped in lymphoid follicles may be pivotal in the development of lymph node pathology and defects in antigen presentation in AIDS (14). Relationships between antibody profiles and the route of infection (23) and the different HIV-1 proteins (13) have been described, but the mechanisms underlying these relations remain unclear. Studies of these phenomena could benefit greatly from analysis of the in situ distribution and kinetics of specific antibody-forming cells (AFC) in biopsy or autopsy material from patients or animals, such as chimpanzees infected with HIV-1 or macaques infected with SIV.

In earlier studies we demonstrated that antigen-specific antibody-forming cells and immune complexes could be demonstrated in vivo by antigen–enzyme conjugates (29). In these studies we used intact proteins, thereby making an analysis of the epitope specificity of antibody-forming cells or immune complexes impossible. Because certain epitopes of proteins clearly are of more interest than others [e.g., molecular mimicry with selfdeterminants (14), virus-neutralizing epitopes], the presence of antibodies directed against these selected epitopes is of great importance.

The aim of this study, therefore, was to develop a method to study the epitope specificity of B-cell responses against HIV-1, or other antigens, in tissues of HIV-1-infected patients or animal models. To this end, two peptides homologous to conserved regions of the HIV-1 gp160 and gp120 were produced by solid-phase synthesis. After immunization of mice with the free peptide or a peptide–carrier conjugate, B-cells producing antibodies against the synthetic peptide could be demonstrated by incubating cryostat sections with a peptide–alkaline phosphatase conjugate. Immunocytochemical double staining permitted the simultaneous detection of isotype and peptide specificity.

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Materials and Methods

Animals. Female BALB/c mice were bred at the TNO breeding facility. Animals 8–16 weeks old were used for immunizations and were kept under GLP protocol with free access to pelleted food and acidified water (pH 3). Experiments were performed under the auspices of Dutch Veterinary Inspection, as described in the law on Animal Experiments.

Peptide Synthesis and Purification. Two peptides were synthesized by solid-phase synthesis according to Merrifield (18) on polystyrene resin (1% cross-linking), using an automated peptide synthesizer (SAM-2; Biosearch, San Rafael, CA). The reaction sequence was performed according to the standard protocol using tertiary-butyl-oxycarbonyl amino acids with the following side chain protection: Lys-2-chlorocarbobenzoxy, Glu-benzyl, Gly-benzyl, Cys-tertiary-butylmercapto (Fluka AG, Buchs, Switzerland and Bachem AG, Bubendorf, Switzerland). Final deblocking and cleavage from the resin was performed by treatment with thioanisol and hydrogen fluoride for 90 min at 0°C. Resin, thioanisol, and cleaved peptide were separated by ether extraction followed by extraction with 5% acetic acid. After lyophilization, peptides were reduced with 10 equivalent threo-1,4-dimercapto-2,3-butanediol, pH 8, for 1 hr and lyophilized. Gel filtration of the crude peptide was performed on Sephadex-G15 (Pharmacia; Piscataway, NJ), using 5% (v/v) acetic acid as the eluent. For analysis by HPLC (high-performance liquid chromatography; Pharmacia), a reverse-phase ultrasphere C18 column, 10 × 250 mm (Beckman Instruments; Palo Alto, CA), was used, applying a linear gradient from 15% to 40% solution B (0.1% trifluoroacetic acid in acetonitrile) into solution A (0.1% trifluoroacetic acid in water) for 15 min at 2.5 ml/min. Amino acid analysis was performed on the hydrolized peptide using pre-column derivatization of the amino acids according to Janssen et al. (11), confirming the expected composition.

Conjugation of Synthetic Peptides. Peptides were coupled to carrier proteins (keyhole limpet hemocyanin, bovine serum albumin, or tetanus toxoid) via the cystein residue with the bifunctional coupling agent MBS, according to a modified method described by Van Denderen et al. (28). A 10 mg/ml solution of the carrier in 0.1 M sodium phosphate (pH 7) was activated at 4°C with a 200-fold molar excess of MBS. After stirring for 15 min, buffer was added to 2.5 ml and the solution was filtrated over a PD10 column (Pharmacia) and concentrated to 2 mg/ml. A 100-fold molar excess of peptide was added for keyhole limpet hemocyanin and a 50-fold excess for bovine serum albumin and tetanus toxoid. The solution was stirred 60 min at room temperature and conjugates were stored at −20°C.

Peptides were coupled to AP by means of glutaraldehyde, with a modification of the method for antibody–enzyme coupling as previously described (7). A 2 mg/ml AP solution was dialyzed overnight at 4°C against 0.2% glutaraldehyde in PBS. After extensive dialysis of the carrier solution against PBS, the peptide was added to the carrier solution (2 mg/ml in PBS) and the mixture was incubated overnight at 4°C. The reaction was stopped with 0.2 M lysine-HCl during 2 hr at 4°C and the mixture was dialyzed against PBS. Conjugates were mixed with an equal volume of glycerol and stored at −20°C.

Immunization Procedures. Mice (n = 16) were primed subcutaneously in the upper side of both hind feet with 25 μg of the peptide or peptide–carrier conjugate in PBS per foot, mixed with an equal volume of Specol, a water and oil adjuvant (5), kindly provided by Dr. A. Bianchi, CDI Lelystad. After 3 weeks the mice were boosted in the same way. Controls were either not treated or immunized with Specol–PBS or immunized with irrelevant peptides/conjugates. Sera taken at time of sacrifice were tested in an ELISA as previously described (4) with a polyclonal goat anti-mouse immunoglobulin antibody (KPL Labs; Gaithersburg, MD) conjugated to AP.

Immunocytochemical (Double) Staining. Mice were sacrificed by cervical dislocation, and spleen and popliteal lymph nodes were snap-frozen in liquid nitrogen. Eight-μm cryostat sections were fixed for 10 min in fresh acetone containing 0.02% (v/v) hydrogen peroxide.

For revelation of anti-peptide AFC only, sections were incubated for 5 hr at room temperature (or overnight at 4°C) with the peptide–AP conjugate diluted 1:25 to 1:125 in 0.1% bovine serum albumin/PBS (w/v). Slides were rinsed thrice with PBS and stained for AP according to Burstone, with modifications (8). Briefly, 5 mg naphthol AS-MX phosphate was dissolved in 250 μl *N,N*-dimethylformamide and added to 40 ml Tris-HCl buffer (0.1 M, pH 8.5). Ten mg Fast Blue BB base was suspended in 250 μl of 2 N HCl, and 250 μl 4% sodium nitrite solution added. After 1–2 min, this mixture was mixed slowly with 40 ml buffer. To inhibit endogeneous alkaline phosphatase activity, 2 mM levamisole was added to the substrate solution. The solution was filtered to remove any precipitate, and the reaction was allowed to proceed for 15–60 min at 37°C by vertical incubation in Coplin jars. Staining was monitored with a light microscope to prevent over- or understaining. Staining was stopped by transferring the slides to PBS. Sections were counterstained with hematoxylin and mounted in glycerol–gelatin.

Double staining was performed in either a one-step or a two-step procedure. In the two-step procedure, sections were incubated overnight at 4°C with unlabeled rabbit anti-mouse isotype-specific antisera (Miles; Weesp, The Netherlands) together with the peptide–AP conjugate diluted 1:250 (approx. 0.04 mg/ml) in 1% bovine serum albumin/PBS. Sections were rinsed three times with PBS and incubated with goat anti-rabbit Ig–HRP (Dakoparts; Glostrup, Denmark), diluted 1:200 in 1% bovine serum albumin/1% normal mouse serum in PBS, for 30 min at room temperature. After washing three times with PBS, AP activity was revealed as described above. Slides were rinsed once and HRP activity was revealed with 3-amino-9-ethylcarbazole. Four mg were dissolved in 250 μl *N,N*-dimethylformamide and added to 9.75 ml sodium acetate buffer (0.05 M, pH 5). Just before use, 50 μl hydrogen peroxide was added. The reaction was monitored with a light microscope and allowed to proceed for 7–10 min.

In the one-step procedure, sections were simultaneously incubated overnight at 4°C with the peptide–AP conjugate and HRP-labeled anti-mouse isotype-specific antisera diluted 1:50 (approx. 0.4 mg/ml) in 1% bovine serum albumin in PBS. Sections were washed three times with PBS, and AP and HRP activities were demonstrated as described above.

Sections were incubated, as a negative control, with unconjugated AP or with AP coupled to a peptide that had not been used for immunization of the animal under investigation. Other controls included spleens of non-immunized mice, and spleen and popliteal lymph nodes of animals immunized with the adjuvant only.

Results

Synthesis of Peptides Homologous to HIV-1 Regions

Two synthetic peptides, SP 68 and SP 69, were produced with Merrifield solid-phase synthesis, and purified. SP 68 is a 21-mer, overlapping the site (marked *) where cleavage of gp160 into gp120 and gp41 occurs (24), with the sequence Thr-Lys-Ala-Lys-Arg-Arg-Val-Val-Gln-Arg-Glu-Lys-Arg*Ala-Val-Gly-Ile-Gly-Ala-Leu-Cys. A carboxyterminal Cys was added for coupling to carrier proteins via −SH residues. SP 69 is a 21-mer homologous to amino acids 254 to 274 of env from the second conserved domain of gp120 and is partially homologous to neuroleukin. Ho and coworkers (10,16) have shown that this region of gp120 is important for HIV-1 infectivity and antibody neutralization. The sequence is Cys-Thr-His-Gly-Ile-Arg-Pro-Val-Val-Ser-Thr-Gln-Leu-Leu-Leu-Asn-Gly-Ser-Leu-Ala-Glu.

ELISA Analysis of Sera

The ELISA data (not shown) confirmed the presence of antibodies in the peripheral blood specific for the synthetic peptide used for immunization. Sera were tested on different coatings and revealed activity against the corresponding peptide, either directly coated as free peptide or coated as a peptide–protein conjugate. Specificity was confirmed by the lack of reactivity against the other peptide (or peptide–protein conjugate) and against irrelevant protein (bovine serum albumin). The tissues used for immunocytochemistry were thus derived from mice that were confirmed to be specifically reactive against the peptide used for immunization.

Immunocytochemical Revelation of Peptide-specific AFC

Coupling of synthetic peptides to proteins can be performed with a variety of coupling agents (3). In this case we chose coupling through the amino groups on both SP and enzyme by means of glutaraldehyde. The conventional glutaraldehyde coupling method (1) is performed by adding the cross-linker to a solution containing both elements to be coupled. This results not only in conjugate formation but also in ineffective homopolymers of both proteins. By modifying a method we described before for the detection of anti-allotype AFC with antibody–enzyme conjugates (7), we succeeded in conjugate formation without any homopolymers. To obtain this, the enzyme is first activated with glutaraldehyde, purified (to remove free glutaraldehyde), and the SP is subsequently added. This results in an SP–AP conjugate with SP bound, in various orientations, through *N*-terminal and ε-amino groups. The conjugates obtained using this new method for coupling of SP to enzymes (proteins) proved very efficient in both immunohistochemistry and ELISA assays (data not shown).

Incubation of cytospin preparations or tissue sections of lymphoid tissues taken from immunized animals will result in binding of these conjugates by extra- or intracellular antibodies present in immune complexes and AFC (Figure 1), respectively. After immunocytochemistry of the SP–AP-incubated samples the AFC will stain blue, as shown in Figure 1a. In spleen sections of immunized mice, 20–80 AFC were detected per section (Figure 1a). Specific AFC were also found in each lymph node section (up to 200/section) after subcutaneous immunization of mice with either conjugated or free SP 68 and SP 69 (Figures 2a–2c). Occasionally a blue-stained honeycomb pattern characteristic for immune complex trapping in lymphoid follicles was observed (Figure 1b). Control sections incubated with unconjugated AP or with the non-corresponding (68-AP on 69 mice and vice versa) peptide–AP conjugate did not show staining of cells. Spleens of untreated mice did not stain with peptide–AP conjugates. Popliteal lymph nodes of mice stimulated subcutaneously with Specol only showed few (10–25) blue-stained cells per section (Figure 2d).

Double Immunocytochemistry for Detection of Isotype and Specificity

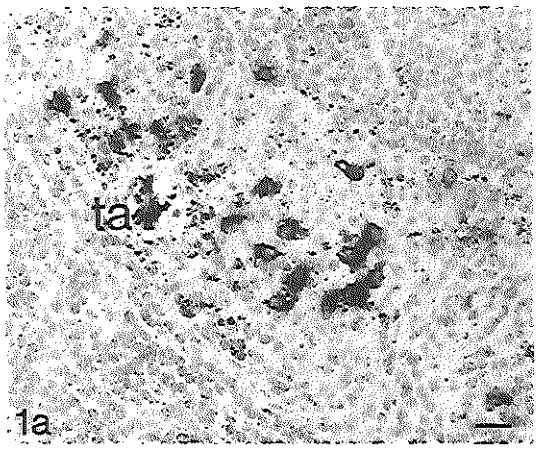
To simultaneously demonstrate peptide specificity and isotype of the AFC, cryostat sections were incubated with both the corresponding peptide–AP conjugate and a rabbit anti-mouse IgG (or IgM)–HRP conjugate. Antibodies recognize the SP and thus bind the SP-AP conjugate (→ blue). These antibodies are also recognized by an HRP-labeled anti-isotype antibody (→ red). As Figure 2 shows in detail, after staining for AP and HRP three types of cells could be distinguished: blue cells producing anti-peptide antibodies of unknown isotype, red cells producing antibodies of the isotype under investigation but not recognizing the peptide, and violet (intermediately)-stained cells producing anti-peptide antibodies of this particular isotype. Double-stained violet AFC-producing IgG (arrows in Figure 2a) and IgM (Figures 2b and 2c) antibodies specifically directed against the peptide were readily detectable. The localization of these AFC in lymphoid tissue was similar to what we have described before (31). AFC were found in the spleen in the outer periarteriolar lymphocyte sheath and in the sheaths of lymphoid tissue surrounding the terminal arterioles (Figure 1a). In the lymph nodes, AFC were found mainly at the boundary of the extrafollicular zone of the peripheral cortex with the deep cortex (Figure 2a) and around the medullary cords (Figure 2b), but only a few cells were found in the follicles. The ratio of peptide-specific AFC of IgM over IgG origin was approximately 2:1, and no differences in localization patterns between the two isotypes were observed.

Discussion

The present study describes a fast and specific approach to analyze the in situ AFC response against synthetic peptides of HIV-1 with respect to localization, epitope specificity, and isotype. This new direct immunocytochemical technique enables in vivo detection of HIV-1 epitope-specific AFC and immune complexes during the development of AIDS. It can be used in the study of several cell phenomena relevant to the histopathogenesis of AIDS (14), which are very difficult to investigate with in vitro methods.

HIV-1 synthetic peptides, and their recognition by antibodies, have been used (in vitro) in serological studies of seropositive subjects (6,12,17) and in functional studies of anti-HIV-1 cell-mediated immunity (2,19,26). We have previously been successful in raising poly- and monoclonal antisera against synthetic peptides homologous to human IgG_2 (4) and the bcr–abl breakpoint region (28) that crossreacted with the native protein. In a parallel line of research, we have developed immunocytochemical methods for detection of AFC specific for protein antigens, such as HSA, and haptens such as TNP and penicillin [for review see (29)]. These methods were used to analyze the development and localization of the humoral response in the murine spleen (30). Combining these two approaches, we reasoned that synthetic peptides, mimicking epitopes of an antigen, could also be used as detector molecules, allowing epitope-specific detection of antibodies directed against that antigen in vivo. Furthermore, synthetic peptides are already established as detector molecules in various assays (e.g., ELISA, immunoblot) as coating for antibody capture from samples such as sera. The experiments described here demonstrate the feasibility of this new application of synthetic peptides as analytical tools in the study of the in vivo immune response.

Immunization of mice with free synthetic peptides (SP 68 and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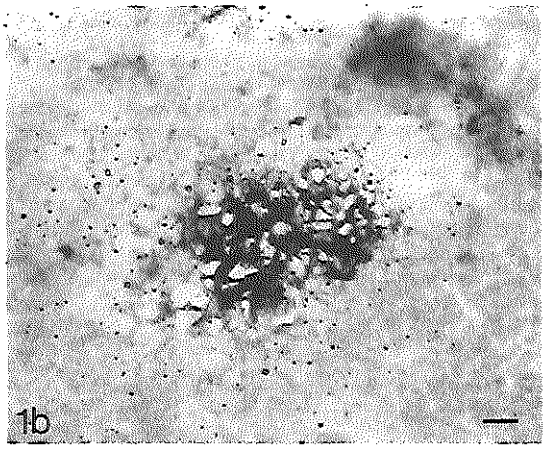
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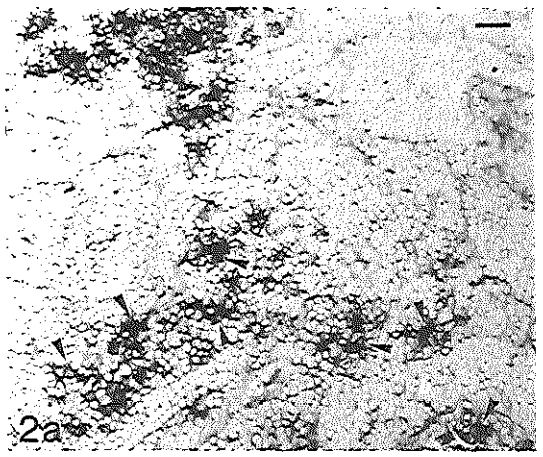
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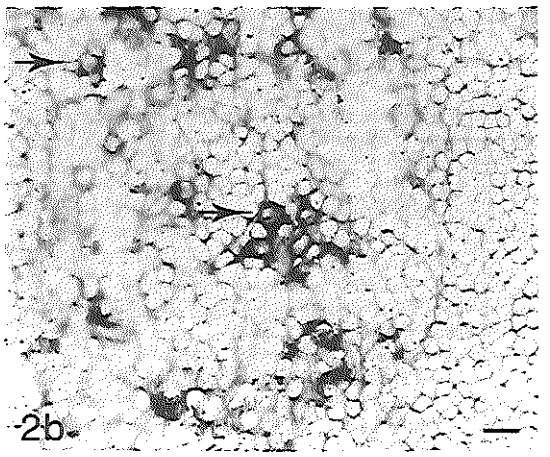
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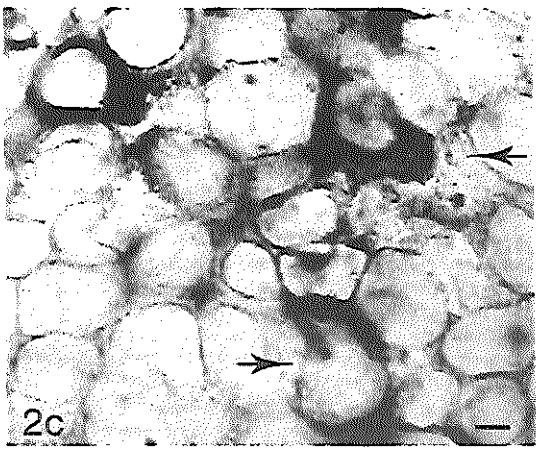
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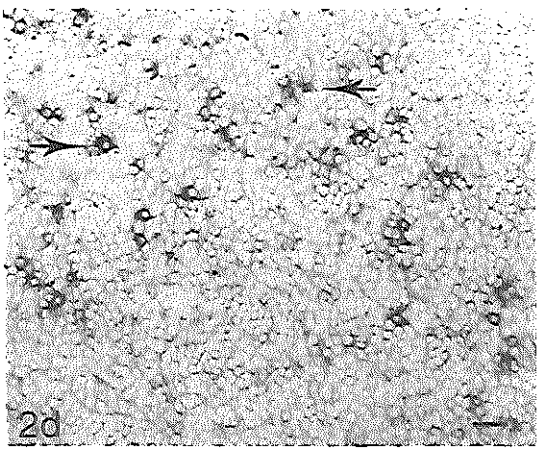
2d

69) or synthetic peptides coupled to carrier proteins led to the development of specific AFC, and these could be detected by incubation of cryosections with a conjugate of SP coupled to a detector enzyme. The different controls (incubation of sections with another peptide–AP conjugate; incubation of sections with unconjugated AP; non-immunized mice; mice immunized with Specol only) confirm the specificity of this method. The incidental peptide-reactive AFC in controls immunized with the adjuvant only may be the consequence of the polyclonal activation by Specol, resulting in B-cells producing crossreactive antibodies. This adjuvant-induced reactivity will, of course, not be present in lymphoid tissue from HIV-1-infected patients. Care should be taken in construction of SP–enzyme conjugates, and especially in validation of their specificity. Too many SP molecules coupled to an enzyme molecule lead to recognition (antibody–SP) but not to staining due to (partial) inactivation of the enzyme. On the other hand, too few SP molecules lead to recognition and staining only of high-affinity antibodies, and preferentially of the IgG isotype. Validation of SP-enzyme conjugates can easily be performed in an ELISA or ELISPOT assay analogous to what we recently described for detection of antibodies directed against insulin with protein–enzyme conjugates (32).

HIV-1 infection and development of AIDS are accompanied by a wide spectrum of immunological abnormalities (15). Although an HIV-1-specific cellular as well as humoral immune response is mounted, little evidence for protective immunity has been found, and it is believed that a large proportion of the infected persons, if not all, will eventually progress to AIDS (21). Abnormalities of the B-cell compartment in AIDS include polyclonal hypergammaglobulinemia, poor in vitro B-cell responses to both T-dependent and T-independent antigens (27), and dramatic changes in number and distribution of B-cells in the lymphoid tissues (22). The present approach enables us to study the HIV-1 specificity, or lack thereof, of these B-cells in vivo. This can be accomplished either by use of SP or by use of HIV-1 proteins such as gp120 (native/recDNA produced).

It has been suggested that other viruses, such as EBV, may be involved in the development of B-cell disorders (15). EBV-specific B-cells can now be studied for the presence of both antigen and specific antibody, by making use of SP derived from EBV sequences or EBV proteins.

Immune complexes that are trapped on the surface of follicular dendritic cells in the lymphoid follicles may be involved in the histopathogenesis of AIDS by acting as a target for cytolytic cells (14). The close intertwining of follicular dendritic cells and surrounding cells poses enormous problems in obtaining intact cells for in vitro studies of the role of immune complexes. Furthermore, the fact that these complexes are usually formed in antibody excess prohibits their detection through recognition of trapped antigen by specific antibodies. The current method, with either SP or protein conjugates, is especially suited for antigen-specific detection of immune complexes by means of the remaining free antigen combining sites (Fab).

Apart from its location in the immune system, HIV-1 has a tropism for the central nervous system and evokes neurological abnormalities (20). The local production of anti-HIV-1 antibodies has been calculated (25) and demonstrated (9), but the origin of these antibodies is unclear. Incubation of cryostat sections of relevant brain tissue with the above-described conjugates would enable the detection of HIV-1-specific AFC in the brain.

By making use of enzymes instead of fluorochromes, we avoid potential problems like autofluorescence and fading of the label. However, if precise quantitation of intracellular antibodies is required fluorochromes can be preferred, as discussed previously (8).

The described method thus provides a tool for studying the fine specificity of the humoral immune response against any antigen in situ, provided that the DNA sequence or amino acid sequence of the antigen is known. Furthermore, many other applications of these SP–enzyme conjugates can be envisaged (e.g., in receptor–ligand studies, ELISA, ELISPOT).

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Figure 1. (a) Cryostat section of spleen from a mouse immunized with SP-68 after immunocytochemistry with 68-AP; note blue-stained AFC around terminal arteriole (TA), and no cells in other compartments. (b) Lymph node section from the same animal demonstrating specific immune complexes. Bars: a = 15 μm; b = 12 μm.

Figure 2. Cryostat sections of lymph node tissue from SP-69-immunized mice (a–c) or adjuvant-only mouse (d), after simultaneous incubation with 69-AP and either anti-IgG–HRP (a) or anti-IgM–HRP (b–d). Note red, blue, and violet (arrows) cells in immunized animals (b, c). In the Specol mouse, localization pattern and normal mouse of IgM plasma cells, but only very few SP binding cells (arrows in d, blue cells), were found. Bars: a = 40 μm; b = 10 μm; c = 5 μm; d = 30 μm.

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Immunocytochemical Detection of Human Immune Deficiency Virus Epitope-Specific Antibody-Forming Cells

Comparisons of Antigens in the Mouse Model

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INTRODUCTION

Infection by human immune deficiency virus type 1 (HIV-1) evokes enormous changes in the organization and function of the microenvironment of lymph nodes and spleen. In three progressive stages, ranging from follicular hyperplasia to lymphoid depletion, the lymph nodes degenerate. In the terminal phase of the disease, the architecture of the lymph node is lost, lymphoid depletion has occurred, and the network of follicular dendritic cells (FDCs) is destroyed (2). The disruption of germinal centers and FDCs (2,3) may be the basis for the development of B-cell disorders (4) in the acquired immune deficiency syndrome (AIDS). Little is known about the mechanisms that govern the progressive damage to the lymphoid organs during the development of AIDS, although they may be the key to HIV-induced immunopathogenesis. Especially, the role of B cells and immune complexes (ICs) during development of histopathogenesis has received little attention. We, therefore, developed an immunocytochemical method to study epitope specificity, isotype and anatomical localization of HIV-specific antigen-forming cells (AFCs) and ICs. By incubation of tissue sections of lymphoid material with synthetic peptides (SP) coupled to detector enzymes, epitope specificity of locally present antibody can be demonstrated. Anti-isotype sera allow double staining for the detection of the isotype (1). In the present study, we determined if recombinant (rec) gp120 could also be used in these techniques, and we compared SP and rec gp120 with HIV- and SIV-infected cell lines as an antigen source.

MATERIALS AND METHODS

Animals

Female Balb/c mice were bred at the TNO breeding facility under specific-pathogen free (SPF) conditions and maintained in Makrolon cages with free access to acidified water (pH 3) and pelleted food. Animals were used for immunization at the age of 12–20 weeks.

Antigens

SP 68, a 21-mer homologous to the cleavage site of HIV-1 gp120 and 41 (amino acids 506–525), was synthesized and purified as described before (1). Rgp120, SIVmac251-infected HUT78 and HIV-1 (HTLV-IIIB)–infected Sup-T1 were a kind gift of Dr. P. Bentvelzen and coworkers (ITRI-TNO, Rijswijk, The Netherlands).

Immunizations

Synthetic peptides: three mice were immunized twice with a 3-week interval in both hind footpads with 25 μg of the SP suspended in Specol, a water and oil adjuvant, in a volume of 50 μl. Rgp120: Two mice were immunized as described for the synthetic peptides but two additional groups were immunized with 50 and 100 μg, respectively. Virus-infected cells were killed by fixation with ethanol (final concentration 70%) and stored at –20°C. Before use, they were washed three times with phosphate-buffered saline (PBS). SIVmac251–infected HUT78: 5 mice were immunized twice with a 3-week interval in both hind footpads with 0, 5×10^2, 5×10^3, 5×10^4 or 5×10^5 cells suspended in Specol in a volume of 50 μl. HIV-1–infected Sup-T1: Four mice were immunized twice with a 3-week interval in both hind footpads with 0, 5×10^3, 5×10^4 or 5×10^5 cells suspended in Specol in a total volume of 50 μl. A second group of four mice received the same doses suspended in Specol in a volume of 200 μl intraperitoneally.

ELISA and Immunocytochemistry

Five days after the booster immunization, serum was taken and tested in ELISA as described previously (5), with a goat-antimouse total immunoglobulin G (IgG)–alkaline phosphatase (AP) (KPL). The spleen and popliteal lymph nodes were taken and snapfrozen in liquid nitrogen. Eight-micrometer cryostat sections were fixed for 10 min in fresh acetone, containing 0.02% H_2O_2. After air drying for 10 min, sections were incubated for 16 hr with antigen-AP conjugates or antigen–horseradish peroxidase (HRP) conjugates (10 μg/ml) at 4°C in 0.1% bovine serum albumin (BSA) in PBS. Conjugates were produced as described previously (1,6). Sections were washed three times with PBS and stained for AP or HRP as previously described (6) and mounted in glycerol/gelatin. Double staining was performed for mice immunized with SP 68 by simultaneous incubation of tissue sections with SP 68–AP and polyclonal rabbit–antimouse isotype–HRP antisera (Miles), specific for IgM and IgG_1. Subsequently, sections were stained for AP and for HRP after washing once with PBS.

RESULTS AND DISCUSSION

The aim of this study was to compare the ability of different types of HIV antigens (i.e., synthetic peptides, rgp120, and virus-infected cells) to evoke antibody responses and to be

Table 1 Application of Different Antigens in Immunization and Detection of Specific B Cells

Antigen	Evokes serum titers	Use as detector ligand
SP 68	Yes	Yes
rgp120	Yes	Yes
SIVmac251–HUT	No	NT
HIV-SuPt1	No	NT

NT = not tested.

used as detector molecules for immunocytochemical detection of AFCs in tissue sections. The results (summarized in Table 1) show that both synthetic peptides and rgp120 give rise to circulating antibodies, and that they can be used as detector molecules in immunocytochemistry. In the dose range used, SIV- and HIV-infected cells do not give rise to anti-gp120 or anti-SP titers.

Figure 1 shows ELISA data of sera tested on coatings of SP and rgp120. It is clear that rgp120 is a potent antigen with the adjuvant Specol (Fig. 1A). The SP 68 is also able to induce specific antibodies (Fig. 1B). Virus-infected cells are not able to evoke responses against either SP or rgp120 (Fig. 1A and 1B). Three alternatives may explain this finding: (a) The quantity of the antigen in the cell preparations is too low. (b) Ethanol fixation has disrupted the antigenic structure of the determinants. (c) Cross reactivity between rgp120 and SIVmac251 glycoproteins is low. The fact that cell preparations in a strong adjuvant are poor inducers of humoral responses may be relevant for vaccination strategies with recombinant prokaryotic organisms which express HIV protein. This problem may be overcome by the use of recombinants expressing high levels of antigen, immunization with higher doses of cells, the use of recombinants replicating in the host, or a combination of these options.

Immunocytochemical staining with rgp120-HRP and SP 68–AP allowed detection of specific AFCs and immune complexes. Figure 2 shows a group of specific AFCs in a lymph node section of a mouse immunized with SP 68. Double staining with SP 68–AP and polyclonal rabbit-antimouse Ig sera coupled to HRP allowed discrimination between (a) antigen-specific AFCs of unknown isotype; (b) AFCs producing antibodies of the isotype under investigation, of unknown specificity; and (c) AFCs specific for the SP, producing antibodies of the isotype under investigation (results not shown). Mice immunized with the adjuvant only showed few or no cross-reactive cells.

CONCLUSIONS

This study shows that both recombinant HIV proteins and synthetic peptides can be used to induce humoral responses in mice. Furthermore, these reagents can be used in immunohistochemistry to detect specific AFCs and ICs in tissue sections of patient and animal material. Such studies will help to elucidate the mechanisms involved in lymphoid histopathogenesis, B-cell (dys)function, and fluctuations of antibody titers. Recently, we showed that both protein-enzyme and peptide-enzyme conjugates can be used successfully for the detection of HIV-specific B-cells in human lymph nodes (7).

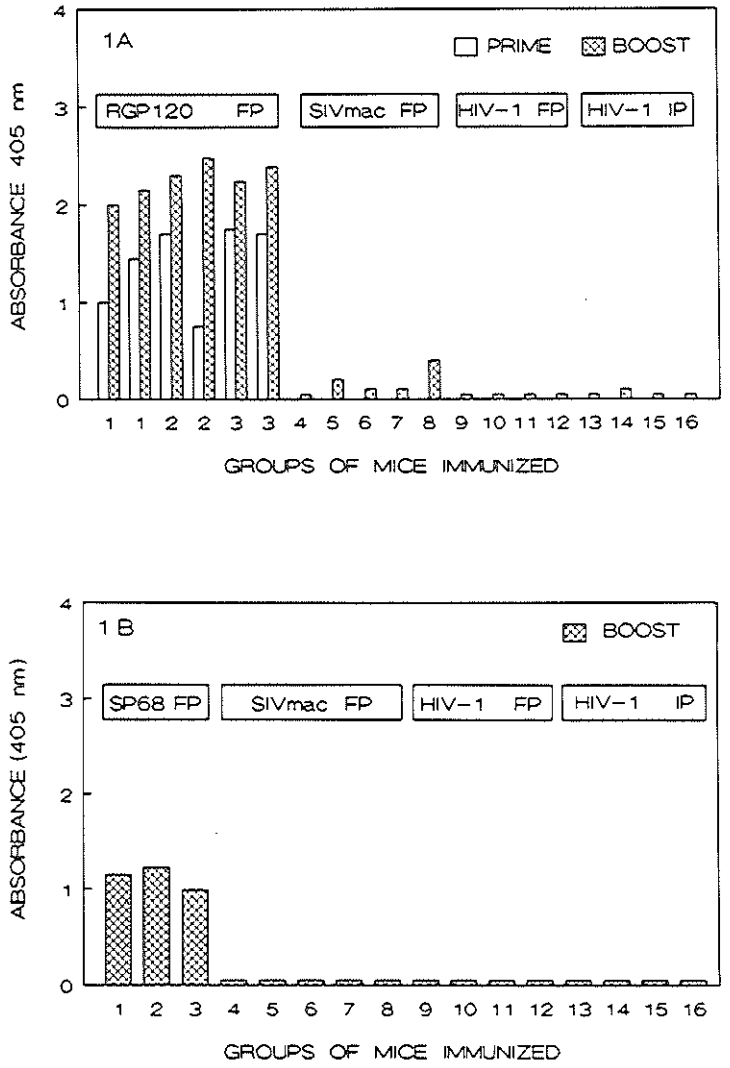

Figure 1 Responses in ELISA of mouse sera after immunization with different HIV/SIV–antigens. 1:100 dilutions of sera are shown. Each bar represents an individual mouse. FP = footpad immunization, IP = intraperitoneal immunization. (A) Evaluation on a rgp120 coat shows that rgp120 induces good responses, whereas HIV-1 and SIVmac–infected cells are ineffective. 1, 2, and 3 represent mice injected with 25, 50, and 100 μg rgp120 per injection, respectively. For rgp120, reactivity of sera three weeks after priming is also shown. 4–8, 9–12 and 13–16 represent increasing doses of virus-infected cells as indicated in the Materials and Methods section. (B) Evaluation on a SP 68 coat shows that the SP induces specific responses, whereas the infected cells induce no antibodies cross reactive with this epitope. 1–3 represent the mice immunized with the SP 68. 4–16 represent the same sera as shown in (A).

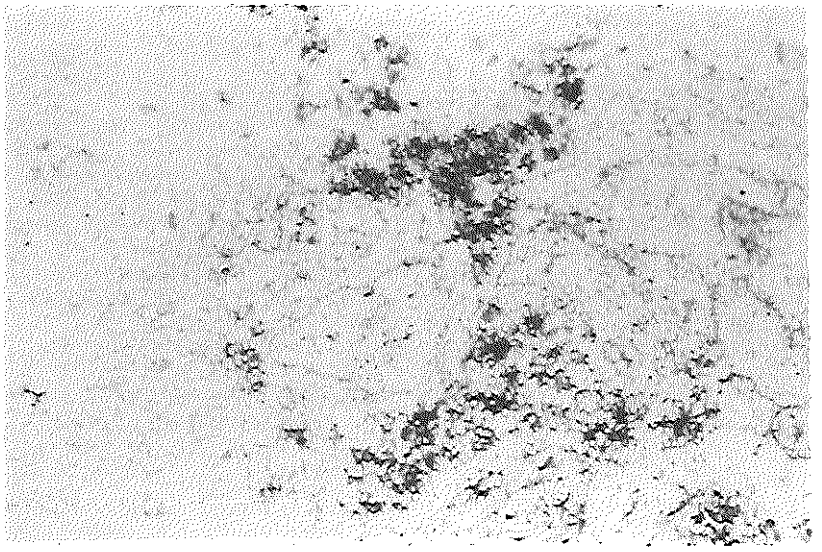

Figure 2 Photomicrograph of a lymph node section of a mouse immunized with SP 68 and stained with SP 68–AP. Darkly stained SP 68–specific AFCs surround a lymphoid follicle.

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Fixation of cryo-sections under HIV-1 inactivating conditions: integrity of antigen binding sites and cell surface antigens

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Summary. Cryostat-sections of biopsies from HIV-infected patients or HIV/SIV-infected experimental animals pose a biohazard risk to laboratory workers. The objective of this study was to select a procedure that appropriately fixes cryo-sections and reduces the risk of HIV-1 infectivity. This inactivation procedure should preserve antigen binding capacity of host-produced antibodies and the antigenic structure of epitopes present in these tissues, while retaining sufficient morphologic detail. We tested the effect of seven different established fixation-inactivation procedures for HIV-1 on the detection of specific antibodies and membrane markers, compared to acetone fixation as a reference. Frozen sections of spleens from mice immunized with trinitrophenyl (TNP)-Ficoll were incubated with TNP-alkaline phosphatase to detect specific antibody-forming cells and follicular immune complexes containing TNP-specific antibodies. In addition, sections were stained with monoclonal antibodies directed against IgM (187-1), T-cells (anti Thy-1), and marginal metallophilic macrophages (MOMA-1). Five procedures proved useful as they gave results similar to regular acetone fixation. In contrast, two procedures with a methanol-containing fixative obscured both antigen binding sites and membrane antigens. Subsequently, these five selected procedures were tested on glass slide preparations of HIV-1 infected cell lines, expressing HIV-1 determinants defined by monoclonal antibodies. Finally, the procedures were tested on sections of an HIV-1 infected human lymph node, for detection of HIV-specific B-cells. We show that fixation-inactivation in 0.37% (v/v) formaldehyde in PBS for 10 min at room temperature and 0.5% paraformaldehyde (w/v) in PBS for 10 min at room temperature are the methods of choice, combining preservation of antigen binding sites (Fab), membrane antigens, and HIV-1 determinants with good tissue morphology.

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Abbreviations: AFC-antibody forming cell; AP – alkaline phosphatase; MAb – monoclonal antibody; HIV-1 – human immunodeficiency virus type 1; HRP – horseradish peroxidase; TNP – trinitrophenyl

Introduction

Immunocytochemical staining of cryopreserved human immunodeficiency virus type 1 (HIV-1) infected tissues poses a biohazard risk, as this virus is not completely inactivated by freezing or by routine 10-min acetone fixation (Fauvel et al. 1989). This problem can be solved by using a fixation procedure which also inactivates virions present in cryostat sections. General procedures for inactivation of cell-free or cell-associated HIV-1 have been described, including treatment with chemical compounds and heat (Martin et al. 1985, 1987; Resnick et al. 1986; Spire et al. 1984, 1985; Hanson et al. 1989). However, a procedure applicable in immunocytochemistry should allow the antigenic structures and antibodies in the tissue to remain intact for subsequent immunocytochemical detection by means of antibody and antigen-enzyme conjugates, respectively. Although the effect of HIV-1-inactivation on antigen detection has been assessed with fluorochrome-labeled antibodies in cell suspensions (Lifson et al. 1986) and cell-smears (Fauvel and Ozanne 1989), these studies did not address the question whether antigen specific antibodies present in tissue sections are still able to bind the complementary antigen.

We have developed techniques to detect specific antibodies in tissue sections with antigen-enzyme conjugates (reviewed by Van Rooijen and Claassen 1986) and used these to investigate the humoral immune response (reviewed by Van Rooijen et al. 1989). Recently, we showed that epitope specificity of antibodies directed against HIV-1 can be determined in tissue sections using synthetic peptide-enzyme conjugates (Laman et al. 1990a; Laman et al. 1991). These studies are based on the use of cryopreserved and acetone-fixed tissues. To reduce the biohazard risk of studies using infected material, it was desirable to find a suitable fixation-inactivation procedure, allowing subsequent immunocytochemistry. It has been described that epitopes on antibodies present in tissue sections are very sensitive to the fixation applied, hampering their detection using isotype-specific antibodies (Boersma et al. 1988, 1989). In addition, detection of specific antibodies of varying affinity in tissue

sections using antigen-enzyme conjugates may be more sensitive to the effects of different fixation procedures than detection of antigens using selected high-affinity antibody preparations (Van Rooijen 1987). The aim of this study, therefore, was to identify a fixation-inactivation protocol for cryo-sections that does not interfere with either antigen-enzyme staining or MAb-staining.

Seven fixation-inactivation procedures described in the literature for effective HIV-1 inactivation were compared to acetone fixation as a reference at three different levels. First, in non-infected murine spleen the following parameters were tested: intracellular antibodies, antibodies present in follicular immune complexes, three MAb-defined cell membrane markers and overall morphology. Second, on slide preparations of infected-cell suspensions, HIV-determinants were detected using MAb's. Third, in a lymph node biopsy of an HIV-1 infected person, HIV-specific B-cells were detected using antigen-enzyme conjugates.

We show that two of the fixation-inactivation methods are detrimental to staining with both antigen-enzyme conjugates and MAb, whereas three give acceptable results and two match the reference.

Materials and methods

Animals

Male (B6D2)F1-mice (Bomholtgård, Rye, Denmark) aged 8–16 weeks, were kept in macrolon cages under an 11 h dark/13 h light regimen at 20° C and were given acidified water (pH 3) and pelleted mouse food (Hope Farms, Woerden, Holland) ad libitum. Experiments were performed under the auspices of the Dutch Veterinary Inspection, according to the law on Animal Experiments. Mice were immunized intravenously with 20 µg TNP-Ficoll, which evokes both IgM and IgG antibody forming cells at the time of sacrifice (Claassen et al. 1986).

Fixation-inactivation procedures

Seven different fixation-inactivation procedures (Table 1) were compared to fixation in acetone containing 0.02% H_2O_2 (10 min) as a reference, a routine method providing a fixation suitable for simultaneous MAb-based and antigen-enzyme immunocytochemical staining (Van Rooijen and Claassen 1986). However, acetone fixation does not inactivate all HIV-1 reverse transcriptase activity (Fauvel and Ozanne 1989). Acetone (pro analysi), formaldehyde, paraformaldehyde and methanol were purchased from Merck (Darmstadt, FRG). Paraformaldehyde was dissolved by warming to 60° C; pH was adjusted with NaOH. H_2O_2 was added to the acetone to inhibit endogenous peroxidase activity (Streefkerk 1972). All procedures were carried out at room temperature. Sections were air-dried after acetone fixation (control), acetone-methanol (I) and acetone-methanol/ethanol/methanol (II). After paraformaldehyde (III) sections were washed once with PBS and adhering PBS was shaken off prior to subsequent acetone treatment.

Antibodies and antigen-enzyme conjugates

Three rat anti mouse MAb (all a kind gift from Dr. G. Kraal, VUA, The Netherlands) specific for murine cell markers were used: anti Thy-1 (59AD2.2) (Ledbetter an Herzenberg 1979) for T cells, MOMA-1 (Kraal and Janse 1986) for marginal metallophilic macrophages and 187-1 for membrane bound IgM. MAb 1044-34 and 1044-13 are murine antibodies obtained through synthetic peptide immunization, and they bind to a determinant on the third variable domain of HIV-1 gp120 (manuscript submitted for publication). CLB 14 is a monoclonal antibody against HIV-1 p24 (Tersmette et al. 1989) and was kindly provided by Dr. M. Tersmette (CLB, Amsterdam, The Netherlands). Optimal dilutions for MAb's were determined by titration (not shown).

TNP-alkaline phosphatase (TNP-AP) was produced as described before (Claassen and Van Rooijen 1984). Recombinant p24 (a kind gift of Transgene, Strasbourg, France) and recombinant gp160 (a kind gift of Pasteur Vaccin, Val de Reuil, France) were coupled to HRP as described before (Laman et al. 1991).

Tissue processing and immunocytochemistry

Mouse spleen tissue: detection of specific AFC and membrane markers. Five days after immunization, the mice were killed by cervical dislocation and spleens were snapfrozen in liquid nitrogen in aluminum containers which were stored at −20° C. Cryostat sections (8 µm) were kept overnight in a sealed box with humidified atmosphere. For detection of TNP-specific AFC and immune complexes in lymphoid follicles, sections were fixed according to the different protocols (s. Table 1), sections were incubated for 16 h at 4° C with TNP-AP (1:125) in PBS containing 0.1% (w/v) BSA. After rinsing three times with PBS, TNP-AP incubated sections were stained as described previously (van Rooijen and Claassen 1986), resulting in a blue precipitate. Briefly, 5 mg naphthol AS-MX phosphate was dissolved in 250 µl *N,N*-dimethylformamide and added to 40 ml *Tris*-HCl buffer (0.1 *M*, pH 8.5). Fast Blue BB base (10 mg) was suspended in 250 µl 2 *N* HCl, and 250 µl 4% sodium nitrite solution was added. After 1–2 min, this mixture was added to 40 ml buffer. To inhibit endogeneous alkaline phosphatase activity, 2 m*M* levamisole was added to the substrate solution. The solution was filtered to remove any precipitate, and the reaction was allowed to proceed for 30 min at 37° C by vertical incubation in coplin jars. Staining was stopped by transferring the slides to PBS. Slides were counterstained with hematoxylin and embedded with glycerol-gelatin.

For detection of membrane markers, sections were fixed (Table 1) and incubated for 1 h at room temperature with the appro-

Table 1. Fixation-inactivation procedures used

Fixation-inactivation		Minutes	Reference
R	Acetone + 0.02% (v/v) H_2O_2 (reference)	10	Van Rooijen and Claassen 1986
I	Acetone-methanol 1:1 + 0.02% (v/v) H_2O_2	20	Fauvel and Ozanne 1989
II	Acetone-methanol 1:1 + 0.02% (v/v) H_2O_2	20	ibid
	Followed by 70% ethanol	10	
	Followed by methanol	10	
III	0.1% (w/v) Paraformaldehyde in PBS (pH 7.5)	20	ibid
	Followed by acetone + 0.02% (v/v) H_2O_2	20	
IV	0.37% (v/v) Formaldehyde in PBS	10	Lifson et al. 1986
V	0.37% (v/v) Formaldehyde in PBS	30	ibid
VI	0.5% (w/v) Paraformaldehyde in PBS (pH 7.6)	10	ibid
VII	0.5% (w/v) Paraformaldehyde in PBS (pH 7.6)	30	ibid

Table 2. Staining intensities of TNP-AP and monoclonal antibodies after different fixation-inactivation procedures

	TNP-AP		MOMA-1	THY-1	IgM⁺ B cells		Morphology	Total
	Intra	Extra	MMMφ	T cells	Cytoplasm	SIgM		
R	++	++	++	++	+±	++	+±	+±
I	±	−	−	±	+	−	+±+	±
II	−	−	−	−	−	−	+++	−
III	+	−	+	+	±	+	±++	±
IV	++	+++±	++	++	−+	++±	−+	++
V	+±	±	+·	++	++	+	−+	+
VI	++	+	±+	++	+±+	++±	++	+
VII	+	+	+++	++	+±	+	+±	+

Sections were assigned a semi-quantitative label, compared to the acetone-fixed control (R) (++ by definition) on the following scale: − = negative, no staining at all; ± = poor staining; + = acceptable; ++ = good, comparable to control; +++ = better than control. TNP-AP staining is differentiated in intracellular antibodies in specific plasma cells and antibodies present in immune complexes trapped in lymphoid follicles. Anti-IgM staining is differentiated in intracellular IgM of plasma cells and SIgm of resting cells. Morphology of the tissue sections is differentiated for the AP and the HRP staining. Overall performance of the procedures is indicated in the last column

priate dilution of the antibody in PBS containing 0.1% (w/v) BSA. After rinsing three times with PBS, a Rabbit anti-Rat IgG horseradish peroxidase (Dako, Glostrup, Denmark) conjugate in PBS containing 0.1% BSA and 1% normal mouse serum was applied for 30 min at room temperature. After rinsing three times with PBS, horseradish peroxidase activity was revealed with DAB. Briefly, 20 mg 3,3'-diaminobenzidine-tetrahydrochloride was dissolved in 40 ml *Trix*-HCl (0.05 *M*, pH 7.6). Just before use 50 µl 3% H_2O_2 was added. The slides and substrate were placed in coplin jars and the reaction was allowed to proceed for 10 min. Slides were counterstained with hematoxylin and embedded in glycerol-gelatin.

Infected cell line: detection of HIV-1 antigens. Aliquots (10 µl) of Sup T1 cells (10^7 cells/ml) infected with HIV-1 (strain IIIB) in vitro were allowed to dry on glass slides prior to fixation-inactivation. After fixation-inactivation according to procedures R, III, V and VII, they were stained with MAb's 1044-34 and 1044-13 specific for a gp120 linear determinant and a MAb (CLB-14) specific for p24 for 1 h, followed by incubation with rabbit anti-mouse IgG AP (Boehringer, Mannheim, FRG) and amplification with APAAP (alkaline phosphatase-anti alkaline phosphatase). Staining was performed with BCIP (5-bromo-4-chloro-3-indolylphosphate) and NTB (nitro blue tetrazolium) as a substrate, producing a black precipitate. Procedures I and II were not applied here in view of the preceding results of the murine tissue; IV and VI were not applied here because the longer incubations (V and VII) gave acceptable results.

Human lymph node biopsy: detection of HIV-1 specific AFC. Fresh cryo-sections were made of a lymph node biopsy (code 388/89) known to contain p24- and gp160-specific B-cells (Laman et al. 1991). After fixation-inactivation according to procedures R, and III to VII, sections were stained with p24-HRP, gp160-HRP and unconjugated HRP as described before (Laman et al. 1991). Procedures I and II were omitted in view of the preceding results of the murine tissue.

Results

Mouse spleen tissue: detection of specific AFC and membrane markers

The results of immunocytochemical staining with an antigen-enzyme conjugate (TNP-AP) and different MAb's after fixation-inactivation of cryo-sections are shown in Table 2. Antibody forming cells and immune complexes were found in the same amounts and localization pattern as described before (respectively: Claassen et al. 1986; Laman et al. 1990b). Although acetone-methanol (I) and acetone-methanol followed by ethanol and methanol (II) gave excellent tissue morphology (Fig. 1), the antigenic profile of the tissue changed drastically, thereby making immunocytochemistry impossible. The combination of paraformaldehyde and acetone (III) resulted in good morphology and acceptable staining of intracellular antibodies and membrane markers, but obscured specific antibody trapped in splenic follicles in the form of immune complexes. The free antigen binding sites present in these immune complexes can normally be demonstrated by incubation with labeled antigen. Formaldehyde in PBS (0.37% v/v) for 10 min (IV) showed good morphology, good detection of intracellular antibodies and membrane markers, and excellent detection of immune complexes and surface IgM. Incubation with this fixative for 30 min (V) resulted in strongly reduced signals for immune complexes and surface IgM. Paraformaldehyde in PBS (0.5% w/v) for 10 min (VI) resulted in good tissue morphology and staining for T cells and marginal metallophilic macrophages, excellent B cell staining but a reduced signal for immune complexes. Incubation with this fixative for 30 min (VII) reduced the detectibility of intracellular antibodies in B cells.

Infected cell line: detection of HIV-1 antigens

Detection of the MAb-defined determinants on infected cells was comparable to the acetone reference procedure (R) for procedure VII, and gave somewhat lower signals for III and V (Fig. 2). In addition to the procedures described here, the MAb-defined determinants are resistant to the fixative normally used for these suspensions, e.g. acetone-methanol (1:1) for 10 min, followed by 70% ethanol for 30 min (results not shown). It should be not-

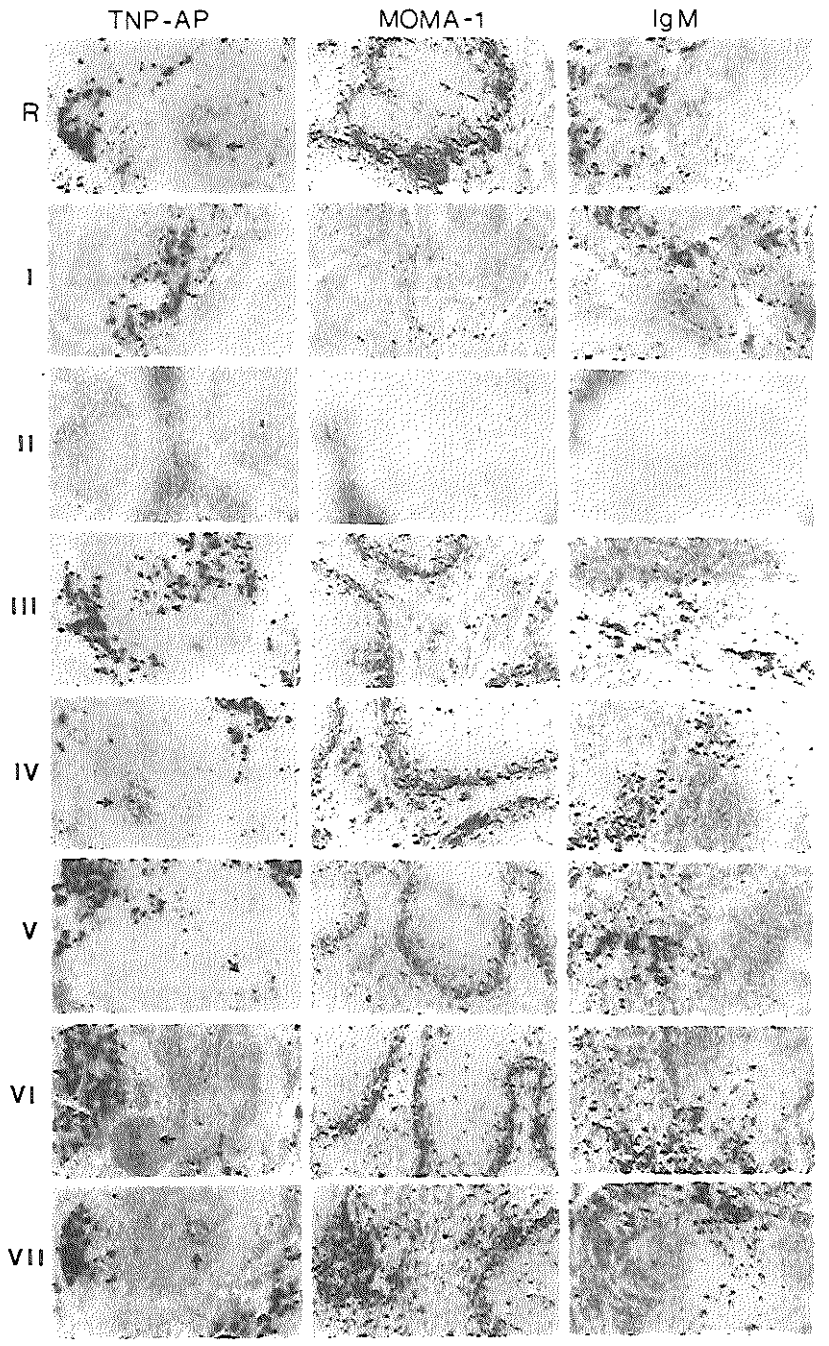

Fig. 1. Immunocytochemistry of murine spleen sections after different fixation-inactivation procedures. Three stainings are shown for all procedures (refer to Table 1). *Left column:* TNP-AP staining for TNP-specific antibody forming cells and immune complexes. Arrows indicate follicular immune complexes. *Middle column:* MOMA-1 staining for marginal metallophilic macrophages. *Right column:* 187-1 staining for IgM. *Bar* in lower right panel indicates 100 μm. See 'Results' section for details

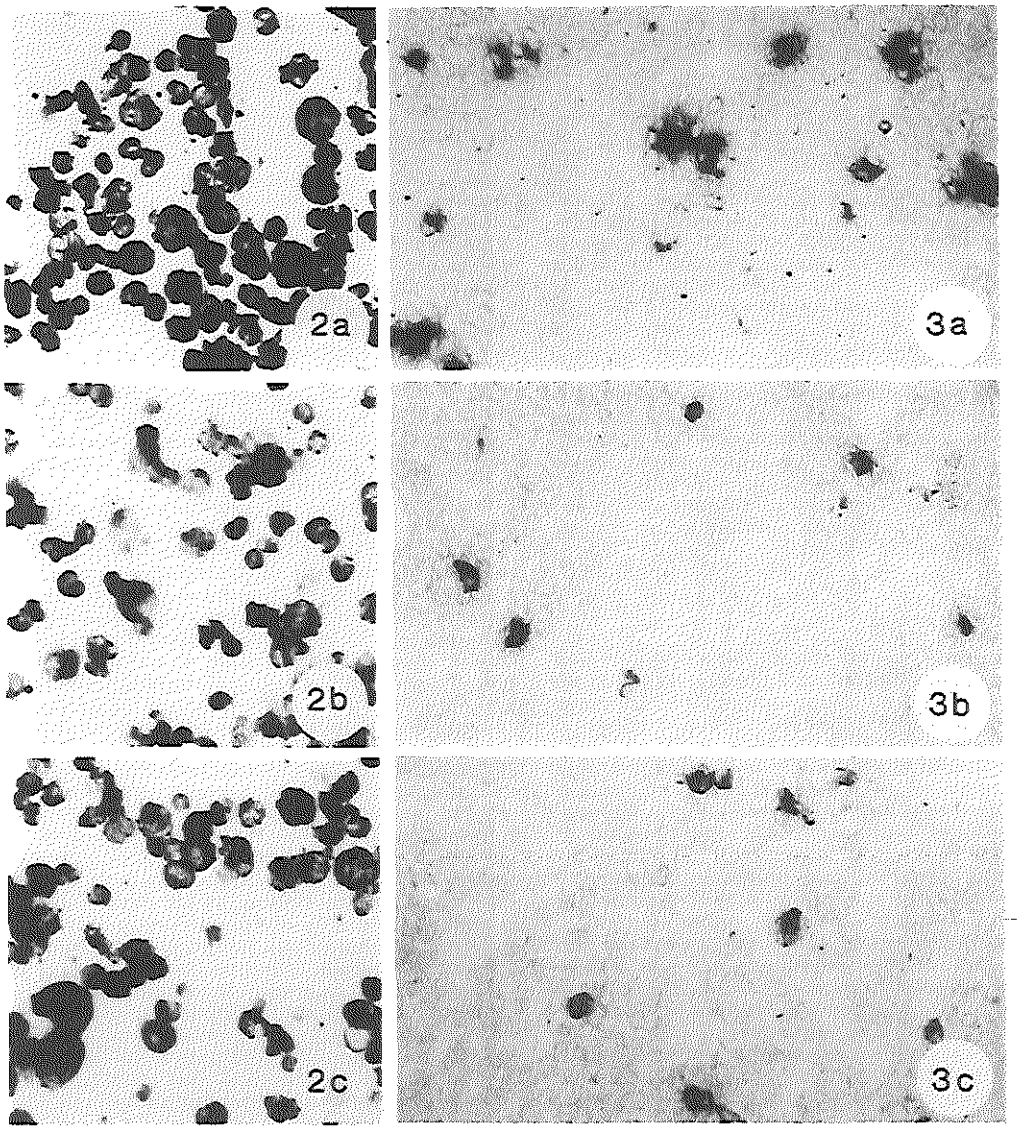

Fig. 2a–c. Immunocytochemistry of cell line preparations after different fixation-inactivation procedures. After fixation-inactivation, sections were incubated with MAb 1044-34, followed by a rabbit anti-mouse IgG HRP conjugate, to detect the expression of the HIV-1 gp120 third variable domain. a acetone fixation (R) b 30 min formaldehyde (V). c 30 min paraformaldehyde (VII). Note that for the stainings illustrated in Figs. 2 and 3 distinct substrates have been used (producing a red and a black color, respectively; see 'Materials and methods'), resulting in different staining intensities. See 'Results' section for details

Fig. 3a–c. Immunocytochemistry of human lymph node biopsy sections after different fixation-inactivation procedures. After fixation-inactivation, sections were incubated with p24-HRP to detect p24-specific antibody and stained with AEC. a acetone fixation (R). b 10 min formaldehyde (IV). c 10 min paraformaldehyde (VI). See 'Results' section for details

ed that the MAb's 1044-13 and 1044-34 recognize a linear determinant, as they have been generated with a synthetic peptide. Linear determinants are expected to be more resistant to denaturation than discontinuous epitopes, which rely on the three-dimensional folding of the protein.

Human lymph node biopsy: detection of HIV-1 specific AFC

B cells containing antibody specific for HIV-1 p24 could be detected in numbers comparable to the reference procedure after IV, VI and VII (Fig. 3), while V resulted in some loss of signal. After procedure III, specific cells could still be found, but in lower numbers than after R. However, faintly-staining gp160-specific B cells could only be detected after R, and lower numbers after III. Procedures IV to VII resulted in a marked increase of the endogenous peroxidase activity of granulocytes, complicating the interpretation of staining results. This problem may be solved by addition of H_2O_2 to the fixatives to inhibit endogenous peroxidase activity. In addition, after fixation with formaldehyde or paraformaldehyde, sections of the human lymph node biopsy get a fatty appearance and seem to dehydrate quickly (see also Figs. 3a–c): it is therefore important to start washing steps and incubations quickly.

Discussion

In this study we show that 0.37% formaldehyde (v/v) (10 min) and 0.5% (w/v) paraformaldehyde (10 min), described in the literature to inactivate HIV-1 (cf. Fauvel and Ozanne 1989; Lifson et al. 1986), can be used to fix cryo-sections without interfering with antigen-enzyme staining for specific antibodies and MAb-staining for membrane markers and an HIV-1 determinant. Three other methods are useful, although staining intensities may be reduced compared to acetone fixation.

Cryostat-sections of biopsies from HIV-infected patients or HIV/SIV-infected experimental animals pose a bio-hazard risk to laboratory workers. Standard acetone fixation for 10 min prior to immunocytochemistry as used in many laboratories is not sufficient to eradicate all HIV-1 activity in contaminated specimens (Fauvel and Ozanne 1989). A procedure which inactivates HIV-1 in sections before, during or directly after fixation would therefore be useful to restrict the infectious potential of the tissue. Heat-inactivation of HIV-1 (30 min at 56° C) is effective (Spire et al. 1985), but is not suitable for tissue sections as it would negatively influence antigenicity and morphology. HIV-1 can be inactivated by chemical compounds such as sodium hypochloride, alcohol, acetone/methanol, quaternary ammonium chloride, lysol, Nonidet-P40, glutaraldehyde, formaldehyde and paraformaldehyde (Fauvel and Ozanne 1989; Hanson et al. 1989; Lifson et al. 1986; Martin et al. 1985, 1987; Resnick et al. 1986; Spire et al. 1984, 1985). For this study, we chose seven rapid and simple fixation-inactivation procedures, which were expected to preserve antigenic structure and tissue morphology.

Our results clearly show that 10 min incubations with formaldehyde and paraformaldehyde give satisfactory results that even exceed the reference for some of the parameters tested in murine spleen tissue (Table 2). Formaldehyde (IV, V) may be preferred over paraformaldehyde (VI, VII) as the detection of antigen-binding sites in immune complexes is considerably better. Longer incubation times with formaldehyde and paraformaldehyde or paraformaldehyde followed by acetone give lower signals than the control for some parameters (Table 2), especially the detection of antibodies present in follicular immune complexes. However, incubations for 30 min with formaldehyde or paraformaldehyde perform well for the MAb's, and may thus be useful for special applications. Fauvel and Ozanne (1989) showed that acetone-methanol (I in this study), acetone-methanol followed by ethanol and methanol (II) and paraformaldehyde followed by acetone (III) resulted in lower signals in immunofluorescence assays. Lifson et al. (1986) showed that 0.37% formaldehyde in PBS performs well as a fixative for detection of cell surface antigens by flow cytometry. These findings are confirmed by our immunocytochemical data.

It is worth noting that extracellular antibodies in follicular immune complexes are more susceptible to fixation-effects than intracellular antibodies in specific B cells (first two columns Table 1). This effect may be a consequence of antigen-enzyme based staining: detection of immune complexes is based on the presence of free antigen binding sites in the complexes. Necessarily, only one antigen binding site (Fab) per antibody molecule will be available for binding of the conjugate, since the other Fab is bound to the antigen in the immune complex (Van Rooijen et al. 1989). In contrast, no antigen is present in antibody-forming cells, so that both antigen binding sites of intracellular antibodies are available for the conjugate. This difference may influence both the amount of conjugate bound and the avidity of the binding. Alternatively, the differences between intra- and extracellular antibodies may result from their accessibility to the fixative. In comparison with the other fixations including the reference, 10 min formaldehyde fixation (IV) shows better detection of extracellular antibodies. It is not clear by what mechanism formaldehyde promotes the detection of extracellular antibodies but a possible explanation is that extracellular antibodies are immobilized through the crosslinking effect of formaldehyde (Brandtzaeg 1982). Experiments performed to detect MAb-defined HIV-1 antigens in infected cell line suspensions and HIV-specific AFC in a human lymph node biopsy after different fixation-inactivation procedures, yield essentially the same conclusions as the murine tissue. However, faintly staining AFC specific for gp160 detected in lymph node sections after acetone fixation are no longer detectable after most other procedures. Possibly, gp160-antibodies in these B cells have lower affinity than the p24-antibodies, resulting in a higher susceptibility to denaturing conditions. Also, the human lymph node sections are more prone to morpho-

logical changes after formaldehyde (IV, V) and paraformaldehyde (VI, VII) treatment (Fig. 3a–e) than the murine tissues. However, these changes do not prohibit the identification of specific AFC.

A major point emerging from the data presented in this study is that the three-dimensional structure of the antigen-binding site of both murine and human antibodies in tissue sections is resistant to the denaturing effects of several fixatives.

It is clear that fixation-inactivation as described here is no guarantee for sterility of sections because residual HIV-1 activity was not assessed, but these procedures do add an additional level of safety. Effectivity of inactivation protocols is strongly dependent on the system used (e.g. cell free virus vs. infected cells) as was shown by Aloisio and Nicholson (1990) in their study of HIV-1 inactivation by 1% paraformaldehyde solutions. Care should be taken in preparation, storage and use of fixative-inactivators to prevent them from becoming stale, inactivated or diluted (refer to WHO guidelines, anon., 1989).

In conclusion, we have shown that several procedures are suitable for fixation-inactivation to reduce the biohazard risks of HIV-1 infected frozen tissues used in immunocytochemistry. These procedures allow subsequent immunocytochemical detection of specific antibodies, cell surface antigens and virus determinants. They may prove useful in daily practice in histopathological laboratories.

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Synthetic peptide conjugates with horseradish peroxidase and β-galactosidase for use in epitope-specific immunocytochemistry and ELISA

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Synthetic peptide-alkaline phosphatase conjugates can be used to detect the epitope specificity of (i) antibody-forming cells in vivo by immunocytochemistry; (ii) of antibody secreting cells in vitro by spot-ELISA; and (iii) antibodies in solution by capture ELISA. The availability of synthetic peptide-enzyme conjugates using detector enzymes other than alkaline phosphatase would offer several important advantages, for example in double staining approaches.

This paper reports the production of synthetic peptide-horseradish peroxidase conjugates and synthetic peptide-β-galactosidase conjugates. A peptide of 21 amino acids (SP 29) was coupled to peroxidase in seven differing molar ratios of peptide over peroxidase, ranging from 1:3.4 to 1:575, using periodate oxidation of the enzyme. SP 29 was coupled to β-galactosidase in four molar ratios ranging from 1.25 to 10, using glutaraldehyde pre-activation of the enzyme. The enzyme activity of the different conjugates was determined, the conjugates were tested in direct capture-ELISA with peptide-specific monoclonal antibodies, and the conjugates were tested in immunocytochemistry to detect peptide-specific B cells. The results show that the conjugates perform best if the peptide is coupled to the enzyme at relatively low molar ratios (1–30). The availability of these new peptide-enzyme conjugates broadens the applicability of synthetic peptides for detection purposes in several assay systems.

Key words: Synthetic peptide; Epitope; Peroxidase; β-Galactosidase; Alkaline phosphatase; Periodate; Glutaraldehyde; Conjugation; Immunocytochemistry; ELISA

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Abbreviations: AEC, 3-amino-9-ethyl carbazole; AFC, antibody-forming cell; AP, alkaline phosphatase; β-Gal, β-galactosidase; BSA, bovine serum albumin; ELISA, enzyme linked immunosorbent assay; HRP, horseradish peroxidase; MAb, monoclonal antibody; ONPG, *o*-nitrophenyl-β-D-galactopyranoside; OPD, *o*-phenylenediamine-dihydrochloride; PBS, phosphate buffered saline; SP, synthetic peptide.

Introduction

In previous work we have used synthetic peptide-alkaline phosphatase (SP-AP) conjugates for immunocytochemical detection of antibody-forming cells in cryostat sections. This technique, which was developed with alkaline phosphatase (AP) as the detector enzyme, permits epitope-specific analysis of antibody responses in experimentally (Laman et al., 1990) or clinically (Laman

et al., 1991) derived lymphoid tissue. The use of synthetic peptides in antigen-based immunocytochemical analysis of antibody production in vivo complements other approaches which use whole proteins or haptens (reviewed by Van Rooijen et al., 1989). Such conjugates can also be used in ELISA (Gerritse et al., 1991), and in spot-ELISA (Sedgwick and Holt, 1986; Vos et al., 1990). The technical possibilities of such studies would be significantly broadened by the availability of conjugates made with detector enzymes other than AP, such as peroxidase (HRP) and β-galactosidase (β-Gal).

The aim of the current study was to develop appropriate SP-HRP and SP-β-Gal conjugates. These conjugates can be useful in double labelling experiments when studying reactivity against overlapping epitopes or determining the isotype of specific antibodies produced, and may offer advantages in situations where there is high endogenous AP activity in the tissue under investigation (e.g., kidney and gut). SP-AP conjugates were produced by glutaraldehyde activation of ε-amino groups of the AP using a dialysis method. This method was also used to produce SP-β-Gal conjugates. HRP, however, is an enzyme encapsulated by a large carbohydrate shell and the availability of ε-amino groups is low, prohibiting the use of glutaraldehyde as a coupling agent. We therefore chose to use the periodate oxidation method (Wilson and Nakane, 1978) to produce reactive aldehyde groups in the carbohydrate shell. An additional advantage of this approach may be that HRP activity is better preserved by not using the few available ε-amino groups from the protein core for coupling.

For both HRP and β-Gal, conjugates with different molar ratios of peptide over enzyme were produced. Evaluation of enzyme activity and testing in ELISA and immunocytochemistry showed that conjugates made using the lower molar ratios of SP over enzyme performed best.

Materials and methods

Animals and immunization

Female BALB/c mice were bred at the TNO breeding facilities. Animals 8–16 weeks old were used for immunization and were kept under GLP protocol with free access to pelleted food and acidified water (pH 3). Experiments were performed under the auspices of the Dutch Veterinary Inspection, as described in the law on Animal Experiments. Animals received an intraperitoneal injection with 15 μg SP 29 in the water and oil adjuvant, specol (Bokhout et al., 1981), mixed 11:9 (v/v) with PBS. A second injection with 50 μg was given 8–15 months after the first. 5 days after the booster injection, spleens were taken and frozen by immersion in liquid nitrogen. Serum was used for ELISA analysis.

Model peptide

SP 29 (Boersma et al., 1989) was produced by Merrifield solid phase synthesis procedures (Merrifield, 1963) as described previously (Van Denderen et al., 1989). This 21-residue peptide is derived from the hinge region of human IgG2 and has the following amino acid sequence: DKTVERKCCVECPPCPAPPVA (MW 2394 Da). SP 29 induces antibody responses in BALB/c mice without coupling it to an immunogenic carrier, and monoclonal antibodies (MAb) binding to different regions of the peptide are available (Boersma et al., 1989).

Peptide-AP conjugation

SP 29 was coupled to AP (MW 65 kDa, Sigma, St. Louis, MO) by means of the glutaraldehyde dialysis method (see Claassen and Adler, 1988). Briefly, 2.5 mg AP PBS was dialyzed overnight at 4°C against 0.2% glutaraldehyde in PBS. After extensive dialysis of the activated AP against PBS, the peptide was added to the enzyme solution (2 mg/ml in PBS) and the mixture was incubated overnight at 4°C. The reaction was stopped with 0.2 M glycine-HCl during 2 h at room temperature, followed by dialysis against PBS. The conjugate was stored at 4°C. We have observed that poorly soluble synthetic peptides may be coupled to AP in the presence of urea (Gerritse et al., 1991).

Peptide-HRP conjugation

SP 29 was coupled to HRP by means of a modification of the periodate method (Wilson and Nakane, 1978). To determine the optimal

ratio of peptide over HRP during coupling, two series of conjugates were produced: in the first set, the amount of HRP was kept constant while the amount of SP was decreased. In the second set, the amount of SP was kept constant while the amount of HRP was varied. 6 mg HRP (Sigma, Type VI, MW 40 kDa) was activated with 3 mg $NaIO_4$ in 1.5 ml de-ionized, 22 μm filtered water for 20 min at room temperature in the dark. 150 μl ethylene glycol were added. The solution was divided into aliquots (4–400 μl) and mixed with the appropriate volume of SP 29 (20–100 μl from a 10 mg/ml stock solution in PBS). Final volumes (550 μl for the conjugates with constant SP, 237 μl for those with constant HRP) were kept constant by adding buffer. In all cases the pH was adjusted to 9–9.5 with $NaHCO_3$ and solutions were mixed for 2 h at room temperature. 50 μl of sodium borohydride (4 mg/ml in distilled water) were added to the 550 μl aliquots and 25 μl to the 237 μl aliquots followed by stirring for 2 h at 4 °C. 50 μl or 25 μl of 0.2 M glycine in PBS were added, followed by stirring for 1 h at room temperature. Conjugates were dialyzed overnight against PBS at 4 °C, concentrated to 150 μl by means of centrifugation in an Ultrafree-MC concentrator (Millipore), mixed 1:1 with glycerol and stored at −20 °C until use. The theoretical molar ratios of the different conjugates are listed in Table I (see results section).

Peptide-β-Gal conjugation

SP 29 was coupled to β-Gal (*E. coli*-derived β-D-galactoside galactohydrolase, MW 540 kDa, Boehringer, Mannheim, F.R.G.) by means of the glutaraldehyde dialysis method as used for SP 29_{23}-AP. 5.5 mg β-Gal were dissolved in 550 μl water and dialyzed against 200 ml PBS containing 0.2% glutaraldehyde at 4 °C overnight. After extensive dialysis of the activated β-Gal against PBS, the peptide dissolved in water at 5 mg/ml, was added to 0.5 mg of the enzyme in different molar ratios (see Table II). Final volumes were kept constant by adding buffer. Further processing was done as described for SP 29_{23}-AP. Molar ratios of 1.25–10 were chosen since previous experiments (results not shown) had demonstrated that of conjugates with molar ratios of 10–1250, only SP 29_{10}-β-Gal showed good performance characteristics in capture ELISA, while SP 29_{250}-β-Gal and SP 29_{1250}-β-Gal precipitated during coupling.

In addition to the glutaraldehyde activation coupling protocol, SP 29-β-Gal conjugates were prepared by adding MHS (maleimidohexanoyl-*n*-hydroxysuccinimide ester, Pierce, Rockford, IL) to a mixture of β-Gal and the peptide. MHS coupling is effected through free SH through groups and amino groups. MHS was chosen instead of the more commonly used MBS (*m*-maleimidobenzoyl-*N*-hydoxy-succinimide ester) because MHS does not give rise to salt precipitates. The molar ratios of peptide over β-Gal were 10, 50, 250 and 1250, using 0.5 mg of β-Gal. The conjugate made with an SP/β-Gal ratio of 1250 precipitated. The other conjugates showed β-Gal activity comparable to unconjugated β-Gal. However, a capture ELISA with two different SP 29 MAb showed no signal whatsoever and immunocytochemistry was also negative (results not shown). This approach does not yield useful conjugates, possibly because of extensive internal coupling of this peptide containing SH groups of four cysteines, and extensive coupling between the peptide and the β-Gal, disrupting the antibody epitopes.

Determination of HRP and β-Gal enzyme activity in the SP-enzyme conjugates

To determine HRP activity after the conjugation procedure, the SP 29-HRP conjugates were compared with non-conjugated HRP. ^{2}log dilutions of samples (based on HRP concentration) in 20 μl sodium phosphate buffer (0.1 M, pH 6.0) starting at 0.28 μg/ml were mixed with 50 μl of OPD (*o*-phenylenediamine-dihydrochloride; Kodak 1078054) at 2 mg/ml supplemented with 0.5 μl/ml 30% H_2O_2 in sodium phosphate buffer in round bottom 96 well microtiter plates (Falcon 3911). The reaction was allowed to proceed in the dark at room temperature for 10 min. and OD_{450} was read with an automated ELISA reader (Multiskan, Titertek).

To determine β-Gal activity after the conjugation procedure, SP 29-β-Gal conjugates were compared with non-conjugated β-Gal. ^{2}log dilutions of samples (based on β-Gal concentration) in 20 μl sodium phosphate buffer were mixed

with 50 μl of ONPG substrate (*o*-nitrophenyl-β-D-galactopyranoside, Pierce, Rockford, IL) in 96-wells microtiter plates. ONPG substrate consists of 3mM ONPG, 10 mM $MgCl_2$ and 0.1 mM β-mercaptoethanol in PBS. The reaction was allowed to proceed in the dark at room temperature for 30 min and OD_{405} was read with an automated ELISA reader.

Direct capture ELISA

The presence of the SP 29 in the conjugates was detected by means of a capture ELISA with three different SP 29-specific MAb (Boersma et al., 1989) and using MAb of unrelated specificity as negative controls. MAb 25-1.2 (mouse IgG1) and MAb 25–2.1 (mouse IgM) bind to the C terminal part of SP 29. MAb 25–4.1 (mouse IgG1) binds to the N terminal part of SP 29. MAb 239–1.1 (mouse IgG1) is specific for FITC. MAb 24–3.3 (mouse IgG1) is specific for an idiotype determinant of human IgG1 kappa. MAb were coated to the wells of 96-wells microtiter plates at a concentration of 10 μg/ml in PBS in 25 μl/well overnight at 4°C. Non-specific binding sites were blocked with 100 μl of PBS with 0.1% gelatin at 25°C for 15 min. After washing five times with PBS containing 0.05% Tween, 20 μl samples of ^{3}log dilutions of the conjugates in PBS-gelatin starting at 1 μg/ml were incubated for 1 h at 25°C. After washing five times with PB5-Tween, HRP and β-Gal activities were revealed as described in the previous section.

Immunocytochemistry

To detect antibody-forming cells (AFC) containing antibodies specific for SP 29, immunocytochemistry was performed using SP 29-HRP and SP 29-β-Gal conjugates. 8 μm cryostat sections of spleen tissue of immunized mice were kept in a box with humidified atmosphere overnight, air dried for 1 h and incubated overnight at 4°C with SP 29-HRP conjugates diluted to 40, 13.3 and 4.4 μg/ml, based on the amount of peptide added during coupling, in PBS containing 0.1% BSA (w/v). 15 μg/ml was subsequently used as an effective and economical working dilution. SP 29_{23}-AP served as a reference. Spleen sections of non-immunized mice and sections incubated with PBS-BSA without conjugate served as negative controls. After washing three times with PBS, HRP activity was revealed by staining with AEC, resulting in bright red staining, as described previously (Van Rooijen et al., 1989). Sections were evaluated for the numbers of AFC present, as compared with the SP 29_{23}-AP control.

Double staining with SP 29_{23}-AP and SP 29_{23}-HRP conjugates to demonstrate any overlap of binding of these two types of conjugate by AFC was performed by incubation with 15 μ/ml SP-HRP for 2 h at 4°C, followed by simultaneous incubation with both conjugates diluted to 15 μl/ml in PBS-BSA overnight. After washing three times with PBS, staining was performed for AP with Fast Blue BB base, resulting in a blue precipitate, followed by washing once with PBS and staining for HRP with AEC. Incubation with only one of the conjugates, followed by staining for both AP and HRP, served as controls.

SP 29-β-gal conjugates were diluted to 40, 20 and 10 μg/ml based on the amount of peptide added during coupling. β-Gal activity was revealed according to Bondi et al. (1982). Briefly, sections were incubated at 37°C for 40 min with 12.5 μl of solution A (10 mg X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, Boehringer, Mannheim, F.R.G.) in 500 μl dimethylformamide), mixed with 500 μl solution B (7 ml PBS supplemented 1.1 mM $MgCl_2$ and mixed with 500 μl water with 50 mM $K_4Fe(CN)_6$ and 500 μl water with 50 mM $K_3Fe(CN)_6$. Sections were rinsed with PBS, counterstained with hematoxylin and mounted in glycerol/gelatin.

Results

Production of SP 29-HRP and SP 29-β-Gal conjugates

Seven SP 29-HRP conjugates were produced by coupling different amounts of peptide and HRP, either by changing the amount of HRP or the amount of peptide added (Table I). SP 29-β-Gal conjugates were produced by coupling different amounts of peptide to a fixed amount of β-Gal (Table II). The molar ratios indicated in Tables I and II are based on the amount of protein added during coupling. Dialysis of the conjugation mixtures would have removed uncou-

TABLE I

RELATIVE CONCENTRATIONS USED IN PRODUCTION OF SP 29-HRP CONJUGATES BY PERIODATE OXIDATION

Numerical subscripts of the conjugates correspond to the molar ratio of SP over HRP used for coupling. + indicates good performance, ± indicates that conjugate may be useful despite lower signals.

Conjugate	SP 29 µg added	HRP µg added	SP/HRP molar ratio	Overall performance
$SP\ 29_5$-HRP	500	1,800	5	+
$SP\ 29_{23}$-HRP	500	360	23	+
$SP\ 29_{115}$-HRP	500	70	115	±
$SP\ 29_{575}$-HRP	500	15	575	±
$SP\ 29_{34}$-HRP	1.000	500	34	+
$SP\ 29_{17}$-HRP	500	500	17	+
$SP\ 29_{3.4}$-HRP	100	500	3.4	+

TABLE II

RELATIVE CONCENTRATIONS USED IN PRODUCTION OF SP 29-β-GAL CONJUGATES BY GLUTARALDEHYDE PRE-ACTIVATION

Numerical subscripts of the conjugates correspond to the molar ratio of SP over β-Gal used for coupling. + indicates good performance, ± indicates that conjugate may be useful despite lower signals, − indicates poor performance.

Conjugate	SP 29 µg added	β-Gal µg added	SP/β-Gal molar ratio	Overall performance
$SP\ 29_{10}$-β-Gal	22	500	10	±
$SP\ 29_5$-β-Gal	11	500	5	+
$SP\ 29_{2.5}$-β-Gal	5.6	500	2.5	−
$SP\ 29_{1.25}$-β-Gal	2.8	500	1.25	−

pled SP 29 and the final number of SP-molecules actually coupled to a single enzyme molecule were unknown.

Determination of HRP and β-Gal enzyme activity in the SP-enzyme conjugates

To determine whether the enzymatic activities of HRP and β-Gal were affected by the coupling with HRP, a titration series of the different conjugates in a microtiter plate was tested with the appropriate substrate and analysed with an automated reader. No significant differences in enzymatic activity of the conjugates were found when compared with the non-conjugated enzyme (data not shown).

Direct capture ELISA

To determine whether the peptide was present and accessible in the different conjugates, peptide-specific MAb were coated to the wells of microtiter plates in a capture ELISA. The results with MAb 25–2.1 (specific for the C terminal part of SP 29) shown in Fig. 1*a* demonstrate that the

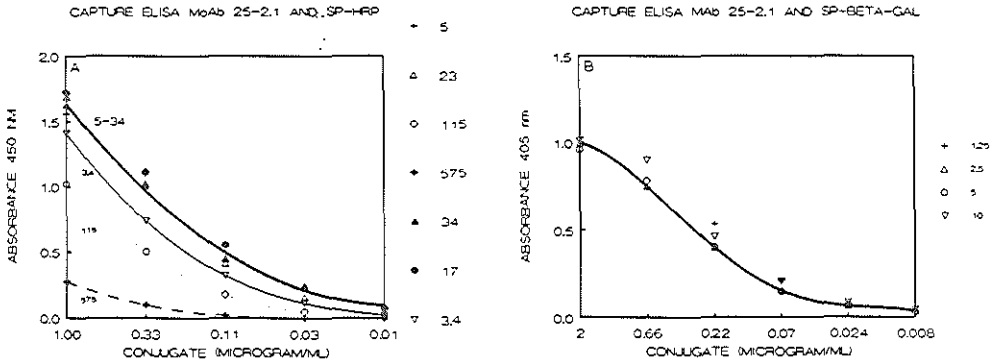
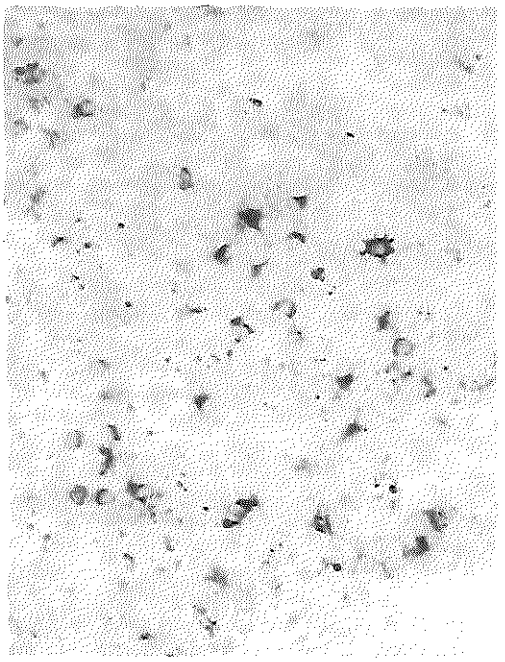
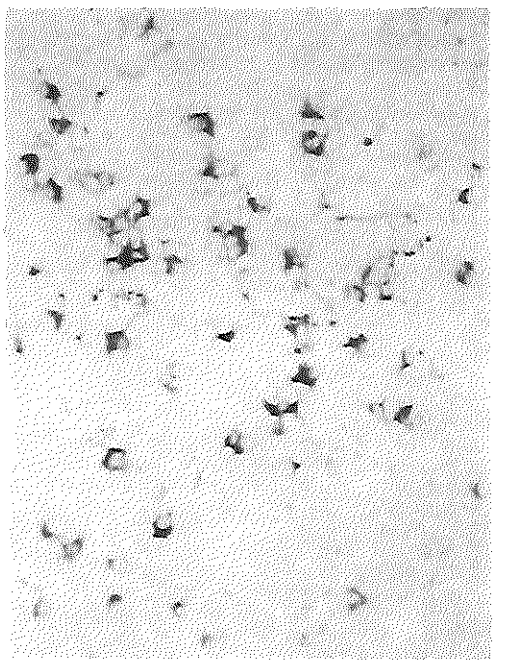
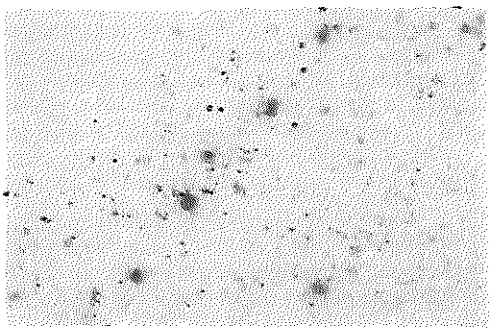


Fig. 1. Direct capture ELISA for detection of SP 29 in SP 29-HRP and SP 29-β-Gal conjugates conjugates. A titration series of the conjugates was tested with SP 29-specific MAb as capture antibody. In the legend, the different conjugates are indicated by their molar SP/enzyme ratios. Results of MAb 25–2.1 (mouse IgM), which binds to the C-terminal part of SP 29, are shown. *a*: SP-HRP conjugates. For clarity, a single line, representing the conjugate with molar ratio 34 has been drawn for conjugates with molar ratios 5, 23, 17 and 34. *b*: SP-β-Gal conjugates. For clarity, a single line, representing the conjugate 2.5 has been drawn.







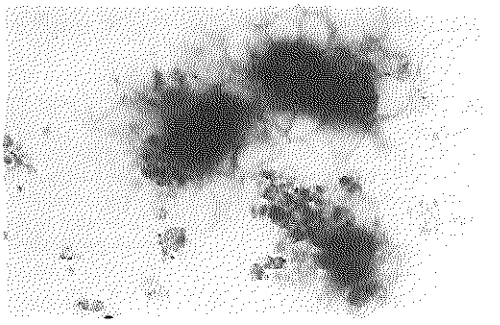


TABLE III

IMMUNOCYTOCHEMICAL DETECTION OF SP 29-SPECIFIC AFC WITH SP-ENZYME CONJUGATES

Numbers of AFC found in frozen spleen sections of mice immunized with SP 29 with SP 29-HRP and β-Gal conjugates were compared with the $SP\ 29_{23}$-AP conjugate. The number of AFC found with SP 29-AP was 100–200 AFC/section (+ + + per definition). 10–100 AFC/section are indicated by + +, 1–10 AFC/section are indicated by +, absence of AFC is indicated by −. A indicates non-specific staining throughout the section.

Immunization conjugate	None	SP 29
None	−	−
$SP\ 29_{23}$-AP	−	+ + +
$SP\ 29_{5}$-HRP	−	+ + +
$SP\ 29_{23}$-HRP	−	+ + +
$SP\ 29_{115}$-HRP	−	+
$SP\ 29_{575}$-HRP	−	+
$SP\ 29_{34}$-HRP	−	+ +
$SP\ 29_{17}$-HRP	−	+ +
$SP\ 29_{3.4}$-HRP	−	+ + +
$SP\ 29_{10}$-β-Gal	−	+
$SP\ 29_{5}$-β-Gal	−	+ + +
$SP\ 29_{2.5}$-β-Gal	A	A
$SP\ 29_{1.25}$-β-Gal	A	A

conjugates made with higher molar ratios of SP over HRP ($SP\ 29_{115}$-HRP and $SP\ 29_{575}$-HRP) gave lower signals while the conjugates made in the range from 3.4 to 34 all performed better and showed the same pattern of reactivity. MAb 25-1.2 (also specific for the C terminal part of SP 29) gave results similar to MAb 25-2.1 (not shown). The negative control antibody 239-1.1 did not bind the conjugates (results not shown). In addition, MAb 25-4.1, specific for the N terminal part of SP 29 did not bind any of the SP 29-HRP conjugates, nor the SP 29-AP conjugate. The SP 29-β-Gal conjugates, made in molar SP/β-Gal ratios of 1.25–10, all reacted similarly with MAb 25-2.1 in the capture ELISA. The N terminal epitope on SP 29, as defined by MAb 25–4.1, could also not be detected in these conjugates.

Immunocytochemistry

To determine whether SP 29-HRP and SP 29-β-Gal conjugates could be used to detect specific AFC in tissue sections, and to determine which conjugates performed best, spleen sections of mice immunized with SP 29 were incubated with the different conjugates. The results obtained are tabulated (Table III) and illustrated in Fig. 2. The numbers of AFC counted correlated directly with the anti-SP 29 serum titers of the SP 29 immunized mice as determined by ELISA (results not shown). It can be seen in Table III that conjugates made at lower SP/HRP ratios performed best in immunocytochemistry. Conjugate $SP\ 29_{23}$-HRP, which performed well in single staining (Fig. 2*a*), was subsequently combined with SP 29-AP (single staining for SP 29-AP shown in Fig. 2*b*) in a double staining procedure. If both conjugates perform similarly, antibodies present in the cytoplasm of any SP 29-specific B cell should be able to bind both types of conjugate, resulting in a violet colour after staining for AP and HRP. The great majority of SP 29-specific B-cells did show this intermediate colour (Fig. 2*c*), demonstrating equal performance of $SP\ 29_{23}$-AP and $SP\ 29_{23}$-HRP.

$SP\ 29_{5}$-β-Gal conjugates could successfully be used in immunocytochemistry, detecting the same numbers of SP 29-specific AFC as AP and HRP conjugates (Table III and Figs. 2*d* and 2*e*). However, the staining of individual AFC was less intense than with AP or HRP conjugates. β-Gal has a tendency to produce precipitates without clear margins (Fig. 2*e*). Also, high-level binding of the conjugate may lead to extensive precipitation of substrate, resulting in dark flushed spots which obscure the cellular aspect (Fig. 2*e*). No

Fig. 2. Immunocytochemical staining of AFC in spleen sections of an SP 29 immunized mouse with different SP 29-enzyme conjugates. *a*: Staining after incubation with $SP\ 29_{23}$-HRP, showing only red cells. ×500. *b*: staining after incubation with $SP\ 29_{23}$-AP, showing only blue cells. ×500. *c*: staining after incubation with both $SP\ 29_{23}$-AP and $SP\ 29_{23}$-HRP showing double stained cells, which indicates that both types of conjugates have been bound by antibodies present in the same AFC. Sections shown in *a*, *b* and *c* all received the complete treatment, with staining for both AP and HRP. ×500. *d*: staining after incubation with $SP\ 29_{5}$-β-Gal, showing blue-green cells. ×330. *e*: detailed view of SP 29-specific AFC, stained with $SP\ 29_{5}$-β-Gal, showing darkly flushed spots without clear cell margins. × 1000.

endogenous β-Gal activity was observed in the splenic tissue using the staining conditions applied here. SP $29_{2.5}$-β-Gal and SP $29_{1.25}$-β-Gal gave strong non-specific staining throughout the splenic tissue. This may be explained by the fact that dilution of the conjugate on the basis of the peptide added can result in high enzyme concentrations. High concentrations of non-conjugated β-Gal do not show this non-specific staining.

Discussion

This study shows that conjugates of synthetic peptides with peroxidase and synthetic peptides with β-galactosidase are useful in immunocytochemistry and ELISA procedures, and can be produced with relatively simple coupling protocols.

We have previously shown that antigen specificity of B cells can be determined in tissue sections by means of antigen-enzyme conjugates, e.g. protein-enzyme or hapten-enzyme conjugates (reviewed by Van Rooijen et al., 1989). We have subsequently extended this type of analysis by developing synthetic peptide-enzyme conjugates. Such conjugates permit the detection of the epitope specificity of antibodies present in the cytoplasm of AFC. In combination with isotype-specific antisera, double staining for epitope specificity and isotype is possible (Laman et al., 1990). These techniques permit the in vivo analysis of specific B cell responses, not only in tissues from experimental animals, but also in lymph node biopsies from HIV-1 infected persons (Laman et al., 1991). The SP-AP conjugates used in those studies were made with the glutaraldehyde dialysis method. In related work, we have shown that synthetic peptides can be coupled to proteins using glutaraldehyde (Zegers et al., 1990) and carbodiimide (Deen et al., 1990). Although these methods were developed for immunization purposes (where the integrity of the carrier protein is of lesser importance), they may also be applicable for enzymes, since the conjugation of enzymes is not in principle different from the conjugation of other proteins.

The availability of conjugates of other detector enzymes (such as HRP or β-Gal) and peptides would extend the potential of SP-enzyme conjugates in the immunocytochemical analysis of epitope specificity. Firstly, immunocytochemical double labelling studies can be performed showing cross-reactivities of antibodies for different peptides, taking advantage of the substrate systems available for other enzymes. Other groups have described the use of β-Gal as a tracer in immunocytochemistry (Bondi et al., 1982), analysis of cell suspensions (Leenen et al., 1987), and in a double staining approach with HRP (Sakanaka et al., 1988).

Secondly, SP-HRP or SP-β-Gal conjugates can be used if high endogenous AP-activity is present in the tissue under investigation, for example in sections of the gastrointestinal tract or the kidney. Thirdly, SP-HRP conjugates can be used for the simultaneous detection of epitope-specifity and isotype, in combination with AP-labeled isotype-specific antibodies.

Apart from their application in immunocytochemistry, SP-AP, SP-HRP as well as SP-β-Gal conjugates can also be used for capture-ELISA and spot-ELISA (plaque-ELISA) systems (Sedgwick and Holt, 1986; Vos et al., 1990). For capture-ELISA, it may be useful to coat the plate with an antiserum to the isotype(s) under investigation, followed by the antibody sample and with the conjugate as the third step. This approach may lead to higher signals, compared with direct coating of the peptide for conventional ELISA, and also to lower background activity. Furthermore, this method permits isotype-specific analysis of peptide reactivity.

Our data demonstrate that coupling of SP to HRP by means of periodate oxidation is feasible and yields the best conjugates at relatively low SP/HRP ratios. Differences in HRP activity of the conjugates are marginal (data not shown), implying that enzyme activity is not influenced by changing the amounts of SP and HRP. An explanation for the lower signals of SP 29_{115}-HRP and SP_{575}-HRP in the capture ELISA may be that high numbers of SP determinants on HRP lead to intensive multiple binding of the conjugate molecules, occupying many antigen-binding sites, and thus limiting the access of other conjugate molecules to the antibodies. No differences in β-Gal activity of the SP 29-β-Gal conjugates were

observed as compared with untreated β-Gal and the differences in reactivity in the capture ELISA were negligible.

Although high SP substitution rates may have a negative effect on conjugate performance, it is clear that multiple binding improves the sensitivity of antigen-enzyme conjugates by increasing the avidity of binding. Therefore, the presence of at least two SP determinants per enzyme molecule is required. Further reduction of SP/HRP ratios may result in conjugates containing HRP with only one or even no SP molecules coupled. In this respect, it is important to realize that coupling of a peptide to a detector enzyme such as HRP is very different from coupling an Ig molecule to HRP (Wilson and Nakane, 1978). In the first case small molecules with low molecular weight (2394 Da for SP 29) are coupled to the 40 kDa HRP moiety, whereas in the second case 40 kDa molecules are coupled to 150 kDa Igs. These considerable differences in size will influence the biological activities of the conjugated molecules, the accessibility of their epitopes and active sites, and their penetrative capabilities. From this point of view, SP-enzyme conjugates may compare favourably with Ig-enzyme conjugates.

The reactive aldehyde groups of HRP induced by oxidation can react with primary amino groups of the protein, which are present in lysine residues and in the N terminal amino acid. The fact that we cannot detect SP 29 in the HRP or the β-Gal conjugates using MAb 25-4.1, may be explained by the presence of two lysine residues and the N terminal amino acid in the epitope recognized by this antibody. Coupling the peptide through these NH_2 groups presumably disrupts this epitope, preventing binding of the antibody specific for this epitope. It should be realized that SP 29 contains many reactive groups (e.g., four cysteine residues with SH groups, amino groups of the lysine and the amino terminus, the terminal carboxyl group and the carboxyl groups of the two glutamic acids residue). For other peptides it may be necessary to re-titrate peptide and enzyme to determine optimal conditions.

The production of SP-β-Gal conjugates seems to be less permissive of variations in the molar ratios of SP and enzyme than the SP-HRP conjugates. This may also be explained by the presence of numerous reactive groups in SP 29. Although SP 29-β-Gal conjugates can be produced successfully and used in immunocytochemistry, the staining pattern of β-Gal precipitates is less than ideal. HRP and AP are clearly to be preferred and β-Gal conjugates may be useful for triple-staining approaches or other special applications. However, a definite advantage of β-Gal is the absence of endogenous enzyme activity under the present experimental conditions.

In conclusion, we have described quick and simple coupling protocols for the production of synthetic peptide-horseradish peroxidase and synthetic peptide-β-Gal conjugates applicable in several immunological assays.

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CHAPTER 6

ANTIBODY FORMING CELLS AND IMMUNE COMPLEXES IN HIV-1 INFECTION

AIDS 1989, 3:543–548

Sir,

Immune complexes on follicular dendritic cells as a target for cytolytic cells in AIDS

HIV infection and subsequent development of immune disorders and AIDS is accompanied by profound degenerative changes in lymphoid tissues, ultimately resulting in massive lymphoid depletion. The mechanisms underlying these changes have yet not been definitively explained, although they are essential to the understanding of the pathogenesis of AIDS which, in turn, is necessary for the development of an effective therapy. We propose here an hypothesis that accounts for the extensive destruction of follicular dendritic cells (FDC) as seen in AIDS.

Lymph-node changes in HIV infection can be divided into three main stages, ranging from hyperplasia to atrophy, which are thought to reflect successive stages of the disease [1]. These stages are: (1) follicular changes, found mostly in persistent generalized lymphadenopathy (PGL), and including lymphadenitis with explosive follicular hyperplasia, increased numbers of CD8+ cells in germinal centers and a moth-eaten appearance of the damaged FDC network; (2) both in PGL and AIDS, follicular hyperplasia and involution can be observed within the same lymph node, follicles show a burned-out aspect, the FDC network being disrupted; (3) lymphadenitis with involuted follicles and lymphoid depletion, which is almost exclusively found in AIDS; only remnants of the FDC network are then present. The majority of the remaining lymphocytes are CD8+.

FDC, in the follicles of lymph nodes and spleen, trap immune complexes (IC) by C3b- and Fc-receptors on their cell membrane. Trapped IC, exposing free antigenic determinants [2], are retained over long periods of time and are involved in the regulation of antibody titers [3] and the generation of B cell memory [4]. They may also function as a persisting antigen reservoir for continuous re-stimulation of antigen-specific B-cells [5]. Destruction of these cells will result in a severely altered lymphoid microenvironment and multiple immune injuries.

Massive destruction of FDC in AIDS as a result of budding of virions, as suggested previously [6], has not been confirmed and is unlikely since only a very small number of lymph-node cells is actually infected [7]. We propose that FDC become targets for cytolytic cells because of the special properties of HIV components in trapped IC.

The presence of HIV particles/antigens in IC between the processes of FDC has been confirmed with electronmicroscopical and immunocytochemical methods [1]. Molecular mimicry between HIV gp41 and MHC class II β 1 domain [8] and between HIV gp120 and class II β chain [9] have been described. These homologous sequences could give rise to a high density of pseudo class II determinants on the FDC through their presence in the trapped IC.

We suggest the following sequence of events in the breakdown of the FDC network: in the early stages of infection, IC formed in the humoral response against HIV localize on FDC. This results in an enhanced expression of MHC class II-like determinants through the presence of gp41 and gp120 in IC. Anatomical co-localization of FDC and CD8+ lymphocytes, lying in gaps of the discontinuous endothelium lining the marginal sinus has been described in people with PGL [10]. These CD8+ cells may exert lysis through: class-I-restricted lysis after recognition of antigen present in IC; antibody-dependent cellular cytotoxicity or lysis after recognition of pseudo class II determinants (altered class II). This lysis will result in retrograde damage to the FDC, gradually dissolving the FDC network and allowing entry of more effector cells. A chemotactical effect of HIV components, which have been shown to possess several different innate biological properties, could also influence the localization of cytotoxic lymphocytes.

The low expression of MHC class II on FDC in combination with the exclusion of CD8+ cells from follicles in healthy individuals may reflect a protective mechanism against autocytolysis.

If our hypothesis is correct, HIV epitopes which induce IC-mediated lysis of FDC should not be included in (subunit) vaccines, so as to avoid damage to these antigen-presenting cells in vaccinated individuals.

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Immunocytochemical determination of antigen and epitope specificity of HIV-1-specific B cells in lymph-node biopsies from HIV-1-infected individuals

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Knowledge about B-cell dysfunction and HIV-specific antibody production is necessary for the understanding of both HIV-1-related immunopathology and the (vaccine-induced) humoral immunity involved in protection against AIDS. This paper describes the application of recently developed methods to detect epitope specificity of B cells in lymph-node biopsies with antigen–enzyme conjugates. Cryosections of five lymph-node biopsies from HIV-1-infected individuals and four control tissues were stained with a panel of HIV-1 antigen–enzyme conjugates: recombinant HIV-1 proteins (gp160, gp120 and p24), labelled with peroxidase, and synthetic peptides representing neutralizing epitopes from gp120 and gp41, labelled with alkaline phosphatase. Antibody-forming cells (AFCs) were detected in all the HIV-1-infected biopsies with gp160, gp120 and/or p24, in numbers up to 350 per section. AFCs producing specific antibodies against peptide 101 (SP 101), representing the neutralizing epitope 586–608 of gp41, were detected in one patient. These techniques allow correlation of *in vivo* function of B cells with lymph-node pathology, clinical stage of the disease and serological data. Their potential for the elucidation of HIV-related immunopathogenesis and the development of vaccines is discussed.

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Keywords: HIV-1, AIDS, lymph-node biopsies, immunocytochemistry, antigen–enzyme conjugates, synthetic peptides, epitope-specificity, specific B cells.

Introduction

Shortly after the recognition of AIDS as a new deficiency of the immune system, it was documented that not only T cells but also B cells showed functional abnormalities [1–3]. In addition to data derived from serology and *in vitro* experiments, histopathological studies described changes in the organization and composition of the microenvironment of lymph nodes [4] and spleen [5,6] in HIV-1-infected individuals. In the majority of lymph nodes with exuberant follicular hyperplasia [4,7], Castleman-like lymphadenitis [4,8–10], and angioimmunoblastic lymphadenopathy-like lymphadenitis [7,9,10], increased numbers of plasma cells and their precursors were found in the follicles or in the pulpa or in both. It is not clear, however, whether these antibody-forming cells (AFCs) produce HIV-specific antibodies or whether they result from non-specific polyclonal activation of the B-cell system.

Although it seems reasonable to assume that a relationship exists between histopathology and *in vitro* functional and phenotypical abnormalities of B cells, there is as yet no satisfactory explanation for either the progressive histopathology or the defects of B cells. Further studies on specific B cells are indispensable as their antibodies are involved in both disease-limiting (virus neutralization) and disease-promoting (Fc-receptor-mediated entry

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[11–13]) effects. The need for more knowledge regarding HIV-specific B cells is emphasized by the fact that most vaccine strategies aim to induce high titers of neutralizing antibodies.

We have recently developed methods to study the specific humoral response *in vivo* by means of antigen–enzyme conjugates [14] and used these to describe the differentiation pathway of specific AFCs in the mouse spleen [15]. The use of HIV-1-derived synthetic peptides coupled with detector-enzymes allowed the detection of epitope specificity of B cells in a mouse model [16]. The application of these techniques to biopsies from infected patients or experimental animals can contribute to the understanding of B-cell abnormalities seen during HIV infection.

The aim of the current study was to show that the application of these techniques on lymph-node biopsies from HIV-infected individuals is feasible and can yield valuable information on antibody production during infection. We show that HIV-1-specific AFCs can be detected with both protein– and synthetic peptide–enzyme conjugates, thus allowing the determination of antigen and epitope specificity.

Materials and methods

Patient materials

Seven lymph nodes were chosen from the biopsy material sent for diagnostic purposes to the Department of Pathology of the Institute for Tropical Medicine, Hamburg, Germany. The material includes lymph nodes of five patients seropositive for HIV-1 and two lymph nodes with follicular hyperplasia not related to HIV infection. In all cases the lymph-node changes were accompanied by a marked plasmacytic reaction. Antibodies to p24, gp41, gp120, and gp160 were detected by Western blot in all the HIV-1-infected individuals. The most relevant clinical and histological data are summarized in Table 1. Two additional negative control tissues were included: a clinically removed human tonsil (designated TVUA) and a spleen from a naive Balb/C mouse (designated Balb).

Antigens

Recombinant HIV proteins were obtained from the following sources: p24 from Transgène (Strasbourg, France); gp160 from Pasteur Vaccins (Val de Reuil, France); gp120 from ABT (Cambridge, Massachusetts, USA) through the Medical Research Council (MRC) AIDS Directed Programme reagent repository (NIBSC, Potters Bar, UK).

p24 (MetAla-p25) is a modified derivative (Pro141-Leu371) of the HIV-1 major core protein produced in *Escherichia coli* and is dissolved in sodium phosphate/NaCl buffer; the sample purity is > 95%. Rec gp120 is produced in a baculovirus expression system and is dissolved in phosphate-buffered saline (PBS); the sample purity is > 90%. gp160 [17] is secreted in the culture medium of BHK 21 cells infected by the recombinant vaccinia virus VV 1163. After purification, the antigen is dissolved in PBS.

HIV-1 synthetic peptides (SPs, listed in Table 2) homologous to neutralizing and/or highly conserved epitopes were selected from the literature and synthesized according to Merrifield solid phase procedures as described previously [22].

Production of antigen–enzyme conjugates

Protein–horseradish peroxidase (HRP) conjugates were produced with the $NaIO_4$-oxidation procedure as described previously [14] in a 1:1 (w/w) ratio of HRP to HIV protein. Synthetic peptide–alkaline phosphatase (AP) conjugates were produced as described previously [16]. Briefly, 2 mg AP was activated by dialysis against 0.2% glutaraldehyde in PBS overnight at 4°C. After extensive dialysis of the carrier solution against PBS, the peptide was added to the carrier solution (2 mg/ml in PBS) and the mixture was incubated overnight at 4°C. The reaction was stopped with 0.2 M lysine-HCl for 2 h at 4°C and the mixture was dialyzed against PBS. Conjugates were mixed with an equal volume of glycerol and stored at −20°C.

Table 1. Lymph-node biopsies in relation to clinical stages (according to the Centers for Disease Control (CDC) of HIV-related disease.

		Blood (cells × 10^6/l)			Lymph-node histology			
Patient	Age/sex	CDC stage	CD4	CD8	Site of biopsy	Type	p24/GC	FDC damage
383/89	47 years/male	IIIB	320	880	Cervical	F	+ +	+ +
388/89	37 years/male	IVB	90	530	Inguinal	AILD-like	+	Only remnants
399/89	34 years/male	(KS) IVD	90	700	Axillary	F	+ + +	+ + +
846/89	25 years/female	IIIB	450	1000	Inguinal	F	+ + +	+ + +
1001/89	32 years/male	IIIB	380	600	Axillary	F	+ + + +	+ +
750/89	49 years/male	Control	NA	NA	Inguinal	F	–	–
1117/89	28 years/male	Control	NA	NA	Cervical	F	–	–

GC, germinal centre; FDC, follicular dendritic cells; F, follicular type; AILD, angioimmunoblastic lymphadenopathy; KS, Kaposi's sarcoma; NA, not analysed. Damage to FDC: – (the network is intact); + + (approximately 25% of the network is destroyed); + + + (up to 75% of the network is destroyed). Score for p24 in GC: + + + + (all GCs are positive); + + + (50% of the GCs are positive); + + (approximately 33% of the GCs are positive); + (one GC or remnants of FDC are positive).

Table 2. Synthetic peptides used for detection of HIV-1-specific B cells.

No.	Protein	Residues	Sequence	Remarks	Reference
69	gp120	254–274	*CTHGIRPVVSTQLLLNGSLAE	Neuroleukin homologue	[18]
98	gp41	519–538	> AVGIGALFLGFLGAAGSTMGA*C	Cleavage site gp120/gp41	[19]
100	gp41	616–632	*CPTNASTSNKSLEQITNN	Neutralizing epitope	[20]
101	gp41	586–608	*CRILAVERYLKDQQLLGIWGCSGK	Neutralizing epitope	[21]
103	gp120	298–314	*CSVEINCTRPNNNTRKSI	Neutralizing epitope V3-domain	[20]

*C indicates a cysteine residue added for coupling purposes; > indicates the gp120/gp41 cleavage site.

Tissue processing and immunocytochemistry

Immediately after surgical removal, the lymph nodes were placed in physiological saline and transported to the department in a container containing ice-cold water. Time differences between removal of the tissue and snap-freezing it in liquid nitrogen were 1–6 h. Material was frozen in plastic airtight containers filled with Tissue-Tek embedding medium and stored at −70°C. Cryosections of 8 µm were cut at −20°C and stored overnight in a box with humidified atmosphere at room temperature. Sections were fixed in fresh acetone (analytical grade), containing 0.02% H_2O_2 to inhibit endogenous peroxidase activity, for 10 min. Sections were air-dried for at least 30 min at room temperature and transferred to PBS (Tris-NaCl) for 30 min. Protein–enzyme conjugates were diluted to 20 µg/ml in PBS with 1% w/w bovine serum albumin (BSA) while SP–AP conjugates were diluted 1 : 100 in PBS/BSA. As negative controls, sections were incubated with PBS/BSA, unlabelled HRP or unlabelled AP. Antigen–enzyme conjugates were incubated overnight at 4°C in a box with a humidified atmosphere.

After washing four times with PBS, HRP activity was revealed by staining with 3-amino-9-ethylcarbazole (AEC) twice for 10 min as described previously [14]. AP activity was revealed with Fast Blue BB salt and AS-MX phosphate. Briefly, 50 mg FBB salt was dissolved in 500 µl dimethylformamide (DMF) and 25 mg AS-MX phosphate was dissolved in 500 µl DMF, and both were added to 50 ml Tris buffer (0.5 M, pH 8.2). Levamisole (20 mg) was added to inhibit endogenous AP activity. The solution was filtered (coarsely) before use. The reaction was allowed to proceed at room temperature for 10 min and was then repeated with fresh substrate after washing the sections once in PBS. This substrate formulation resulted in good specific staining and a significant reduction in endogenous AP activity of human tissue when compared with the procedure used routinely for animal sections [14].

Double staining with gp160–HRP and SP101–AP was performed by simultaneous incubation of the two conjugates, followed by staining twice for AP, washing once in PBS and staining twice for HRP. Control sections were incubated with either PBS/BSA, gp160–HRP or SP101–AP, and were subjected to the same staining procedure. Sections were embedded in glycerol/gelatin either directly or after counterstaining with hematoxylin for 1 min. The number of AFCs per section was determined by counting conjugate-binding cells in non-counterstained sections with conventional light microscopy. After HRP staining, AFCs could be distinguished from granulocytes by the bright-red homogeneous staining of the AFC cytoplasm, leaving a clear white spot representing the nucleus. Endogenous HRP activity in granulocytes was easily distinguishable from specific staining as it gave rise to dark-red clumps of precipitate without cellular aspect. Endogenous AP activity was low and restricted mainly to vessels and posed no problems for the identification of specific AFCs. The surface area of eight sections per biopsy was determined (in mm^2) by computer-aided image analysis (MCID software, Imaging Research Inc., Brock University, St Catharines, Ontario, Canada) of images obtained with an HTHMX-1 camera (High Technology Holland BV, Eindhoven, The Netherlands).

In addition, damage to follicular dendritic cells (FDCs), as well as the presence of *gag* proteins of HIV-1 in germinal centres, was analysed. Air-dried cryostat sections were fixed in acetone for 15 min at room temperature and incubated with monoclonal antibodies specific either for FDCs (DRC-1, Dakopatts, Glostrup, Denmark; dilution 1 : 50) or for HIV-1 p24 (BT3, Biotech, Rockville, Maryland, USA; dilution 1 : 800). We used the alkaline phosphatase AP–anti-AP (APAAP, Dianova, Hamburg, Germany) technique to visualize the antibodies [23].

Results

Lymph-node cryostat sections from HIV-1-infected individuals were probed for the presence of HIV-1-specific B cells using immunocytochemical staining with antigen–enzyme conjugates. To correct for the differences in size of the lymph-node biopsy sections, data are shown as number of AFCs per surface area. The average surface area of a biopsy section was obtained by measuring eight sections by image analysis (see Materials and methods section) and calculation of the average and standard deviation of the mean: biopsy 383/89, 55 ± 1 mm^2; biopsy 388/89, 63 ± 2 mm^2; biopsy 399/89, 33 ± 2 mm^2; biopsy 846/89, 35 ± 1.5 mm^2; biopsy 1001/89, 27 ± 2 mm^2; biopsy 750/89, 25 ± 2 mm^2; biopsy 1117/89, 32 ± 2 mm^2; tonsil TVUA, 53 ± 3 mm^2, and spleen Balb, 7 ± 1 mm^2. The average surface area of lymph-node biopsies is 38 mm^2. Data are expressed as number of AFCs per 40 mm^2.

Immunocytochemical staining with protein–HRP conjugates revealed AFCs specific for gp160, gp120 and/or

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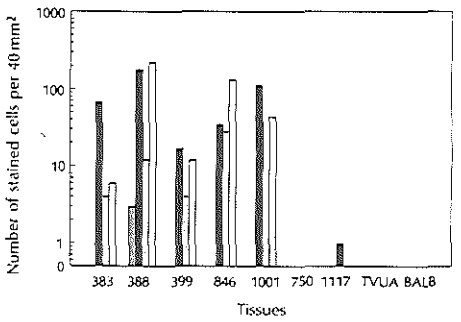


Fig. 1. Presence of antibody-forming cells (AFCs) in biopsies after staining with protein–horseradish peroxidase (HRP) conjugates. Numbers of AFCs per 40 mm² are shown on a log scale. No significant numbers of AFCs were found in the negative controls, whereas all HIV-1-positive lymph nodes contained AFCs producing antibodies reactive with one or more of the conjugates. ▨, phosphate-buffered saline; ▩, HRP; ■, gp160; ▧, gp120; □, p24.

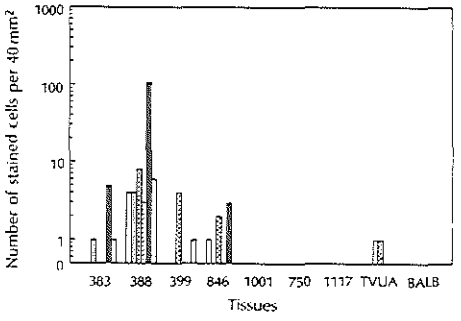


Fig. 2. Presence of antibody-forming cells (AFCs) in biopsies after staining with synthetic peptide–alkaline phosphatase (SP–AP) conjugates. Numbers of AFCs per 40 mm² are shown on a log scale. Biopsy 388/89 shows high numbers of SP101–AP-binding AFCs. ▨, phosphate-buffered saline; □, AP; ▩, SP69; ▩, SP98; ▧, SP100; ■, SP101; ▤, SP103.

p24 in lymph-node sections from all patients infected with HIV-1 (summarized in Fig. 1 and illustrated in Figs 4a, d–f). p24 and gp160-specific AFCs were found in all patients, while gp120 responses were lower or absent (399/89, 1001/89). As can be seen in Fig. 1, up to 350 AFCs specific for an HIV protein (p24) could be found per section. In biopsy 388/89, which shows the strongest response, about 650 AFCs are involved in the anti-gp160 and anti-p24 humoral response.

Immunocytochemical staining with SP–enzyme conjugates revealed 170 B cells specific for SP101 (homologous to a neutralizing epitope of gp41) in a section of biopsy 388/89 (Figs 2 and 4b). To show that these AFCs can bind both the SP and the homologous sequence in the whole protein, we performed a double staining with gp160–HRP and SP101–AP. The results, shown in Figs 3 and 4c, demonstrate that most cells binding the SP101–AP also bind the gp160–HRP, resulting in a violet colour. This is evidence that the peptide as synthesized has the same conformation as the original sequence present in the recombinant gp160.

Low numbers of AFCs binding the SP–AP conjugates were found in several biopsies, but the possible cross-reactivity with AP did not permit the conclusion that these cells were HIV epitope-specific.

AFCs were mostly found around the follicles in the interfollicular zone of the peripheral cortex. Occasionally, some AFCs were found in the follicles. AFCs were distributed singly or in small groups (two to six cells), suggesting clonal development (Figs 4e and f).

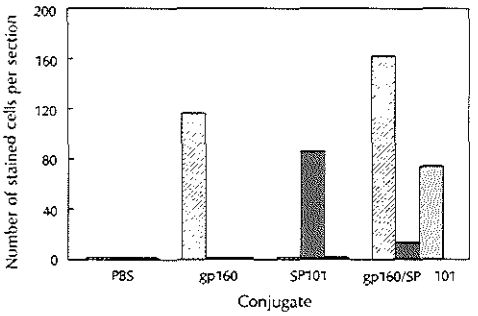
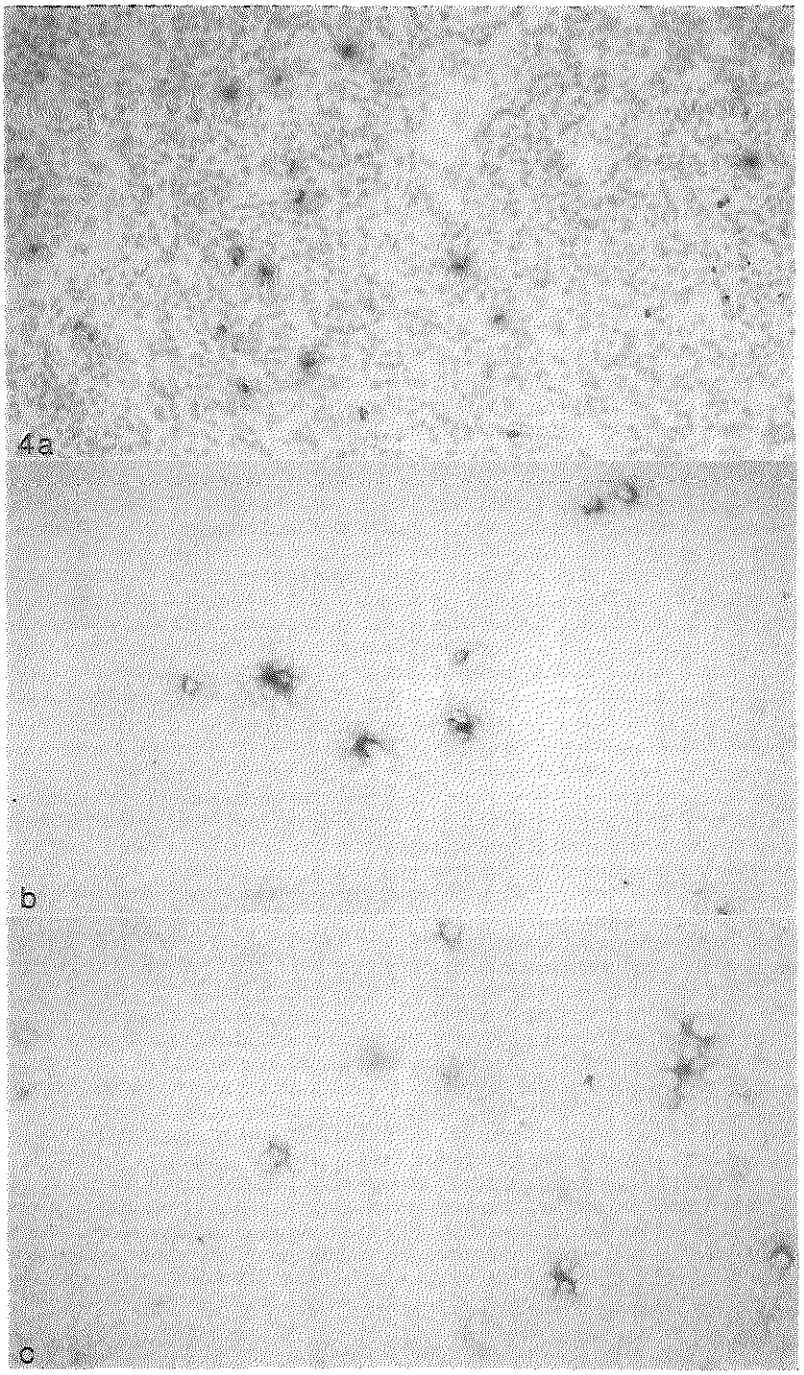


Fig. 3. Presence of antibody-forming cells (AFCs) in biopsy 388/89 after double staining with gp160–horseradish peroxidase (HRP) and synthetic peptide 101–alkaline phosphatase (SP101–AP). Incubations of four sections from biopsy 388/89 with four different conjugate combinations were performed. Numbers of blue, red or violet AFCs per section are shown on a normal scale. The numbers demonstrate that most SP101-reactive cells also bind the gp160, confirming the cross-reactivity between peptide and protein. ▥, red; ▨, blue; □, violet.

No cells cross-reactive with the conjugates were detected in the control tissues. Control incubations with PBS showed very little endogenous HRP activity (except for the red pulp of the mouse spleen), while some endogenous AP activity was present, especially in the blood vessels. Control incubation with unconjugated AP and HRP revealed incidental cross-reactive AFCs in 388/89 (Fig. 1).

Discussion

This study shows that protein and epitope specificity of HIV-1-specific B cells can be determined in tissue sections of lymph nodes from HIV-infected individuals using antigen–enzyme conjugates. This is the first time a humoral HIV-specific response in human lymphoid tissue has been demonstrated *in situ*. Functional abnormalities of the B-cell compartment during HIV-1 infection have been well documented [1–3] and include elevated serum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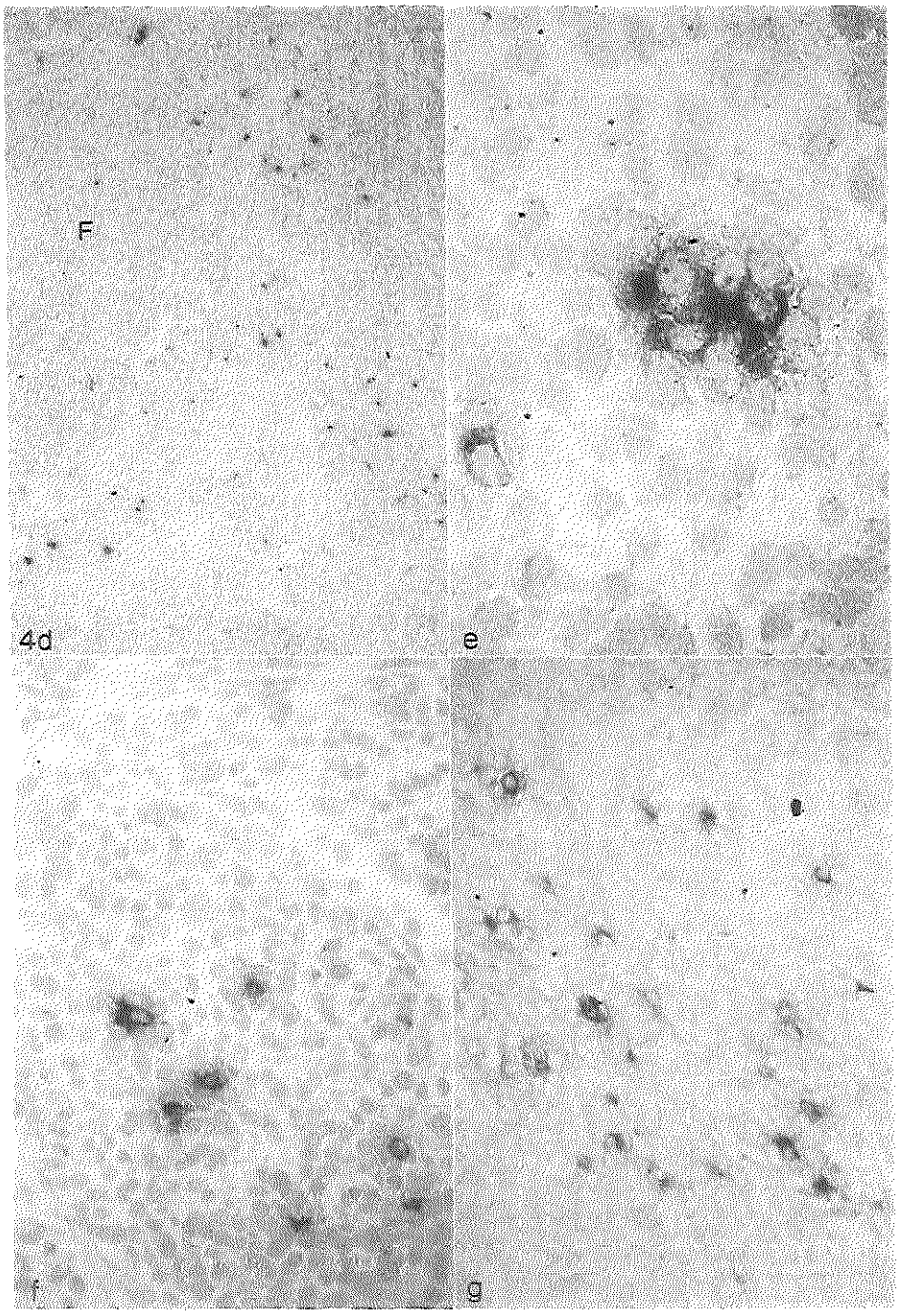
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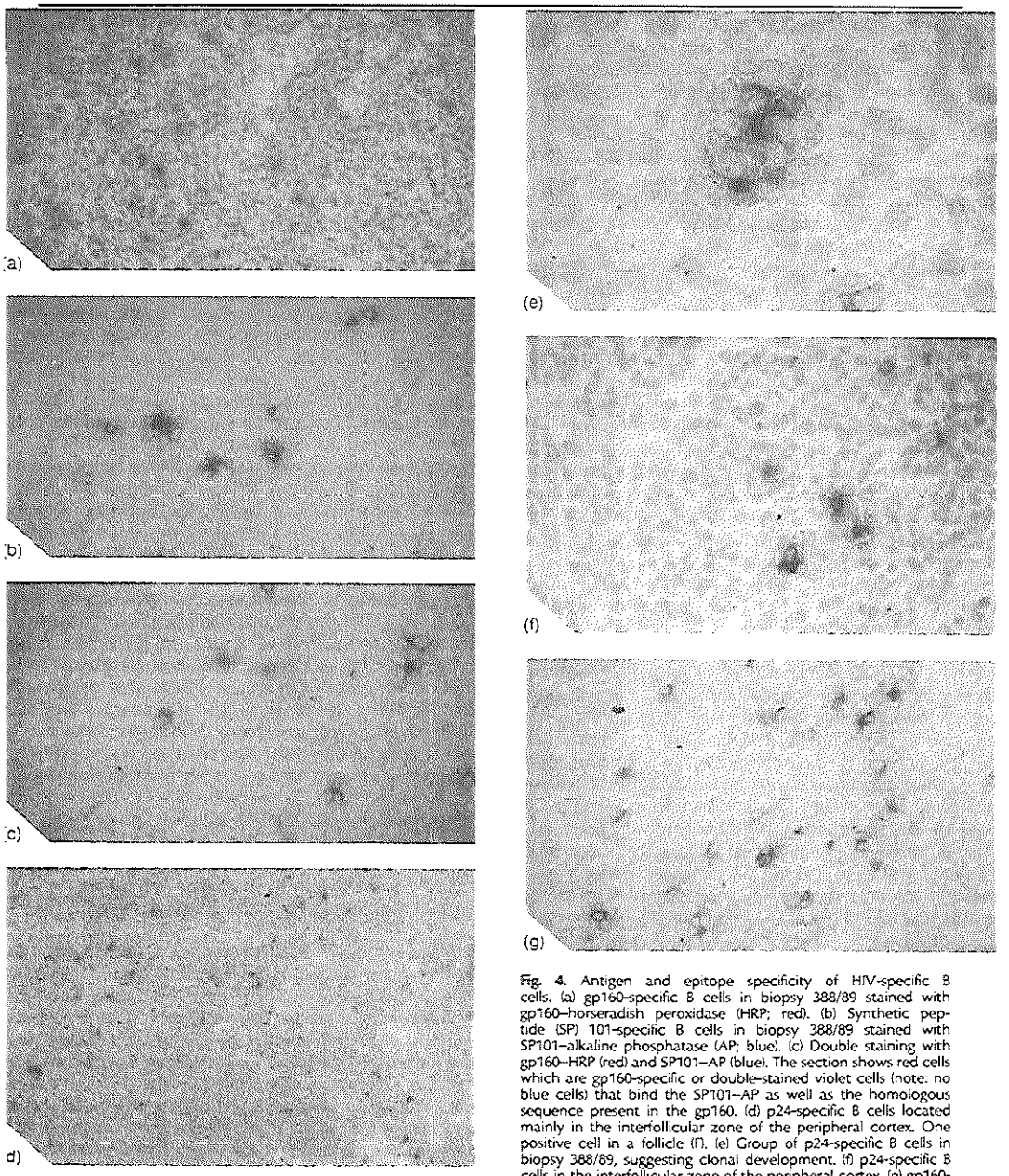

Fig. 4. Antigen and epitope specificity of HIV-specific B cells. (a) gp160-specific B cells in biopsy 388/89 stained with gp160–horseradish peroxidase (HRP; red). (b) Synthetic peptide (SP) 101-specific B cells in biopsy 388/89 stained with SP101–alkaline phosphatase (AP; blue). (c) Double staining with gp160–HRP (red) and SP101–AP (blue). The section shows red cells which are gp160-specific or double-stained violet cells (note: no blue cells) that bind the SP101–AP as well as the homologous sequence present in the gp160. (d) p24-specific B cells located mainly in the interfollicular zone of the peripheral cortex. One positive cell in a follicle (F). (e) Group of p24-specific B cells in biopsy 388/89, suggesting clonal development. (f) p24-specific B cells in the interfollicular zone of the peripheral cortex. (g) gp160-specific B cells localized as in (f).

immunoglobulin levels, presence of circulating immune complexes, inability to mount an appropriate immune response after immunization, elevated numbers of spontaneous plaque-forming cells, enhanced responsiveness to B-cell growth factors, and refractoriness to the normal signals for B-cell activation. There is as yet no satisfactory explanation for the B-cell abnormalities during HIV-1 infection and the fluctuation in titers of antibodies against different HIV-1 antigens.

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Immunocytochemical detection of HIV-specific AFCs in intact tissues complements the serological and *in vitro* data. It has high sensitivity, excludes *in vitro* artefacts and, in general, allows the study of specific antibody production in relation to the lymphoid microenvironment [24].

In the present study, considerable numbers of AFCs producing antibodies specific for p24, gp160 and gp120 were detected in lymph nodes from HIV-infected individuals. The specificity of this reaction was confirmed using HIV-1-negative control tissues and incubations with medium or the uncoupled detector enzyme. The numbers of specific AFCs found are comparable to those found in experimental animals killed at the peak of the response after secondary immunization with protein antigens [14]. In those experimental settings, animals received prime and booster injections with a purified, non-replicating antigen. In contrast, during natural HIV-1 infection in humans, the replicative antigen is continuously present. Apparently, this results in chronic stimulation of B cells followed by production of specific antibody in these patients. Previous investigations have shown deposits of *gag* proteins and the presence of HIV-1 in the germinal centres in association with FDCs of lymph nodes [25] and spleen [7]. It has also been documented by repeated lymph-node biopsy specimens that HIV antigens persist in germinal centres for long periods of time [26,27]. The stimulatory properties of HIV-1 virions, proteins and synthetic peptides *in vitro* have been described [28–31]. It is conceivable that HIV antigens localized in germinal centres have a similar stimulatory effect on germinal centre B cells. In addition, functional alterations of B cells during HIV infection may be related to the progressive destruction of the network of FDCs [25]. These cells are thought to be involved in the presentation of antigen in the form of immune complexes to B cells for the formation of memory B cells [32], the regulation of antibody titers [33,34] and possibly for antibody production [35]. The FDC network is an essential homing and supportive element for B cells, and their destruction (possibly as a result of cytolytic attack [36]) may be crucial to B-cell dysfunction.

The anatomical localization of the HIV-1-specific AFCs around the follicles and distributed more randomly in the medulla is reminiscent of that found in animal lymph nodes [14,37,38]. The finding that specific B cells are often found in small groups may reflect clonal development (Fig. 4e and f).

The chance of finding epitope-specific B cells reactive with the synthetic peptides used here is small. First, the virus is hypervariable, and it is not known with what virus strain the patients are infected and how the epitope is represented in this strain. Second, the number of B cells reactive with a short sequence is smaller than those reactive with the whole protein. Finally, the reactivity against an epitope may be dependent on the stage of the disease. However, with the SP101, representing an extensively described determinant from the immunodominant part of gp41 [21,39–41], we were able to detect specific AFCs in one patient. Double staining with gp160–HRP showed that the peptide-reactive cells cross-reacted with the whole protein, demonstrating that the conformation of the peptide and the corresponding native sequence is identical. The low number of blue, apparently only peptide-reactive, cells found may be the result of stronger binding of the SP101–AP conjugate than the gp160–HRP conjugate. Alternatively, blue-stained cells may result from the presence of AP-cross-reactive antibody. It can also be argued that not all SP101 molecules have the same conformation as the homologous sequence in the intact gp160: some B cells may be specific for this other conformation. There is as yet no obvious explanation for the high reactivity of biopsy 388/89; systematic studies on more biopsies should show whether this is an exception or whether this response is associated with a special histological lymph-node pattern.

The antigen–enzyme technique also allows the detection of free antigen-binding sites present in immune complexes trapped in lymphoid follicles [14]. The lymph nodes used here showed p24 positivity in lymphoid follicles as detected with monoclonal antibodies. We did not detect trapped immune complexes, however, with the p24–HRP conjugate or with any of the other conjugates. The most likely explanation for this finding is that all antigen-binding sites are occupied by antigen, which may be expected to be present in excess. Alternatively, binding of p24–HRP by follicular immune complexes may lie below the detection level. A third possibility is that p24 is present in the germinal centres in uncomplexed form; there is evidence from animal studies that compounds may localize in germinal centres without involvement of antibody [42]. It is important to note that with monoclonal antibodies, gp120 has never been demonstrated in germinal centres, and gp41 only incidentally [43].

We now plan to test routinely all new biopsies for gp120-, gp160- and p24-specific AFCs. In addition, we will use these methods to evaluate the anti-simian immunodeficiency virus response in macaques and the anti-HIV response in chimpanzees in forthcoming experiments

In conclusion, the current study provides a new approach to the study of HIV-1-specific B cells in relation to histopathology and clinical stage of the disease. Considerable numbers of plasma cells in a lymph-node section are involved in anti-HIV responses. The use of synthetic peptide enzyme–conjugates allows epitope-specific analysis of natural or vaccine-induced responses. These methods can be used to study infected human or animal tissues and are applicable for all pathogens from which purified antigens are available or for which DNA or amino-acid sequences are known.

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Reuil, France (rec gp160), Transgene, Strasbourg, France (rec p24) and the MRC AIDS reagent repository of the NIBSC, Potters Bar, UK (rec gp120, produced by ABT, Cambridge, Massachusetts, USA). We also thank Dr N. van Rooijen (Department of Histology, Medical Faculty, Vrije Universiteit, Amsterdam, The Netherlands) for helpful discussions and critical reading of the manuscript.

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CHAPTER 7

GENERATION OF HIV-1 SPECIFIC ANTIBODIES USING SYNTHETIC PEPTIDES

VARIANT-SPECIFIC MONOCLONAL AND GROUP-SPECIFIC POLYCLONAL HIV-1 NEUTRALIZING ANTIBODIES RAISED WITH SYNTHETIC PEPTIDES FROM THE GP120 THIRD VARIABLE DOMAIN

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ABSTRACT

The third variable domain (V3) of the human immunodeficiency virus type 1 (HIV-1) external membrane glycoprotein gp120 is of crucial importance in eliciting neutralizing antibodies in infected persons. Polyclonal (PAb) and monoclonal antibodies (MAb) directed against selected epitopes within the V3-domain are valuable tools to analyse the involvement of such sequences in neutralization and to define the relation between amino acid variability and immunological cross-reactions. The aim of this study was to obtain such site-specific antibodies using the synthetic peptide approach.

We here report on a group-specific neutralizing PAb, two high-affinity IIIB neutralizing MAb and two non-neutralizing MAb, all raised with synthetic peptides derived from the V3-domain. A 15-amino acid peptide overlapping the tip of the V3-domain of HIV-1 MN was used to produce a rabbit PAb (W0/07). This PAb inhibited syncytium formation induced by HIV-1 IIIB and four field isolates.

A similar IIIB-derived peptide was used to generate two murine IgG1 MAb (IIIB-V3-13 and IIIB-V3-34). Pepscan-analysis mapped the binding site of IIIB-V3-34 to the sequence IRIQRGPGR. The K_D of IIIB-V3-13 and IIIB-V3-34 for gp120 was $6.8 * 10^{-11}$ and $1.6 * 10^{-10}$ M, respectively. These MAb neutralized IIIB, but not MN, and inhibited syncytium formation induced by IIIB. They are applicable in ELISA, immunocytochemistry and flow cytometry.

A peptide covering the left base of the V3-domain was used to generate two murine IgG1 MAb (IIIB-V3-21 and IIIB-V3-26). The binding site of IIIB-V3-21 was mapped to the sequence INCTRPN. These MAb did not neutralize HIV-1, and did not inhibit syncytium formation.

This study supports the notion that HIV-1 neutralizing antibodies, suitable for multi-assay performance, can be obtained with synthetic peptides, and that high affinity MAb can be generated. Such site-specific antibodies are useful reagents in the analysis of HIV-1 neutralization. In addition, the cross-neutralization of different viral strains by PAb generated through single-peptide immunization is directly relevant to vaccine development.

INTRODUCTION

The third variable domain (V3-domain) of the HIV-1 external glycoprotein gp120 evokes a major fraction of neutralizing antibodies during a natural infection (Goudsmit et al., 1988; Rusche et al., 1988; Javaherian et al., 1989). The V3-domain is thought to consist of 36 amino acids which form a loop as a result of disulfide-bonding of cystein residues at positions 303 and 338 (Javaherian et al., 1989; Leonard et al., 1990) see also Table 1). The V3-domain seems to be involved in

virus-cell fusion and cell-cell fusion (Freed et al., 1991) and its structural integrity is important for viral infectivity (Kowalski et al., 1987). Fusion may involve cleavage of the V3-domain by cell surface or endosomal proteinase (Clements et al., 1991). The positively charged amino acids concentrated in the V3-region may function in binding and fusion (Callahan et al., 1991). Several studies using monoclonal antibodies have confirmed the role of the V3-domain in viral infectivity (Åkerblom et al., 1990; Durda et al., 1990; Fung et al., 1987; Gorny et al., 1991; Kinney-Thomas et al., 1988; Linsley et al., 1988; Matsushita et al., 1988; Scott et al., 1990; Skinner et al., 1988).

The V3-domain is therefore considered to be an important determinant to include in candidate vaccines. The extreme sequence heterogeneity of the V3-domain in different isolates is a major obstacle in vaccine development, but recent seroprevalence studies provide evidence that certain motifs, especially sequences highly homologous to V3 of the isolate MN, may be relatively conserved (LaRosa et al., 1990; Zwart et al., 1991). Candidate vaccines including such sequences may evoke antibodies capable of neutralizing a wide variety of naturally occurring virus-types (Javaherian et al., 1991).

Monoclonal and polyclonal antibodies are useful reagents to analyse the role of different epitopes of the V3-domain in neutralization and to probe the relation between primary sequence variation of HIV-1 and serological cross-reactivity *in vitro*. In addition, antibodies are important tools in immunocytochemical approaches to elucidate the mechanisms of HIV-1 induced immunopathology *in situ*. We recently described methods to study HIV-1 specific B-cells in tissue sections of lymph node biopsies using antigen enzyme conjugates (Laman et al., 1990b, 1991a 1991c, in press, a). In combination with these techniques, HIV-1 variant-specific antibodies applicable in immunocytochemistry would allow study of specific antibody formation in relation to protein expression and antigen localization of the variant under investigation.

The synthetic peptide (SP) approach is the method of choice to obtain antibodies against pre-selected linear sequences. Most groups use anti-peptide PAb, however, and reports on HIV-1 neutralizing MAb raised with SP of the V3-domain are scarce (Durda et al., 1990). In addition, virus neutralization may be directly related to antibody affinity, a point which is often overlooked. Vaccine-induced circulating antibody should be of high affinity, allowing lower titers to be effective (Layne et al., 1989). Therefore, the aim of the current study was to generate high-affinity neutralizing murine IgG MAb using SP derived from the V3-domain. In addition, we sought to demonstrate the occurrence of antibodies cross-neutralizing different HIV-1 strains after immunization with a single V3-derived peptide to obtain further support for the concept of peptide immunization.

MATERIALS AND METHODS

ANIMALS

Female Balb/C mice were bred at the TNO breeding facilities. Animals 8-16 weeks old were used for immunization and were kept under GLP protocol with free access to pelleted food and acidified water (pH 3). Female Flemish Giant rabbits (outbred) of 6-8 kg were kept under the same conditions and used for immunization at 15 months of age. Experiments were performed under the auspices of the Dutch Veterinary Inspection, as described in the law on Animal experiments.

PEPTIDE SYNTHESIS

The different sequences from the V3-domain to be synthesized were selected from Myers et al. (1990). A terminal cystein residue was added for coupling purposes (see also figure 1). Peptides were synthesized as described in detail before (Zegers et al., 1991) on RapidAmide resin beads using Fmoc protected amino acids (Dupont, USA) following the procedure as described for the RAMPS System (Dupont, Medical Prod., Biotechnology Syst., USA). Peptides were purified using liquid chromatography on G15-Sephadex (Pharmacia) in 5% acetic acid. Fractions were analyzed on a Beckman Ultrosphere 5 μm reversed-phase C18 column using a gradient of acetonitrile with 0.1% trifluoracetic acid. Fractions with the same major compound and with high purity were pooled and lyophilized twice. Amino acid analysis was performed to confirm the composition of the peptides.

CONJUGATION AND IMMUNIZATION

Peptides were coupled to KLH (keyhole limpet hemocyanin)(Calbiochem, San Diego, CA, USA) as an immunogenic carrier by means of MBS (m-maleimidobenzoyl-N-hydroxysuccinimide ester)(Pierce, Rockford, IL) as described in detail before (Boersma et al., 1988). Briefly, coupling was performed by mixing MBS (200 mol/mol carrier protein) and the carrier on ice in PBS. After removal of the excess MBS, the SP (100 mol/mol carrier) was added and the mixture was stirred for 30 min at room temperature.

Groups of three mice received injections of 25 μg of either the free peptide or the SP-MBS-KLH conjugate mixed with Specol (a water-in-oil adjuvant) subcutaneously on the upper side of both hind feet. Antibody responses were monitored with ELISA (enzyme linked immunosorbent assay) on weekly taken serum samples. After 4 weeks mice received a booster injection with the same dose and via the same route. A mouse with a high response on both peptide and recombinant

gp120 was selected for fusion and received two additional injections, intraperitoneally and in the hind feet. Four days after this second booster the spleen was used for fusion.

Rabbit W0/07 was immunized three times with 200 μg of SP 155-MBS-KLH intradermally in four places on the back. The first and second immunization were given at day 0 and day 28 using Freunds complete adjuvant, the third was given at day 52, using Freunds incomplete adjuvant. Serum samples were taken before immunization and two weeks after every immunization. The sample taken after second immunization (first booster) was tested in a syncytium formation inhibition assay (SFI). The animal was bled three weeks after the final immunization.

GENERATION OF MONOCLONAL ANTIBODIES

Fusion was performed according to Haaijman et al. (1988). Briefly, a cell suspension of spleen cells obtained four days after the second boost was mixed with Sp2/0 cells in logarithmic growth in a ratio of 10:1 and incubated with 40% polyethylene glycol (PEG 4000, Merck) and 5% DMSO in PBS for 1 min. After dilution of cells with RPMI they were incubated for 20 min at room temperature and kept overnight in regular medium (RPMI 1640, 10% FCS [fetal calf serum], 2 mM glutamine, 0.1 mg/ml streptomycine, 100 E/ml penicillin, 1mM sodium pyruvate and 0.05 mM beta-mercaptoethanol). Afterwards, they were cultured on selective medium (regular medium supplemented with 1 μg/ml azaserine and 0.1 mM hypoxanthine) and tested regularly with ELISA. After one week of culture the azaserine was discontinued. Subcloning of positive wells was performed by limiting dilution at 0.5 cells/well. Ascites was produced injecting 10^6 hybridoma cells in 0.25 ml PBS intraperitoneally in Balb/C mice. Ascitic fluid was collected under anaesthesia.

ELISA AND AFFINITY DETERMINATION

ELISA was performed as described by Boersma et al. (1988). Briefly, 96-well pvc-roundbottom microtiter plates were coated with 5 μg protein/ml or 10 μg/ml of the unconjugated SP over night at 4 °C. Non-specific binding sites were blocked with 0.5% gelatin in PBS for 30 min at room temperature. Plates were washed with PBS 0.05% Tween 20. Samples were diluted in PBS/gelatin/0.05% Tween 20 and incubated for 1 hr at 25 °C. After washing, a Rabbit anti-mouse IgG-AP (alkaline phosphatase) conjugate (KPL, Gaithersburg, MD, USA) was added and incubated for 1 hour at 25 °C. After washing, AP-activity was determined with PNP (paranitrophenylphosphate; Boehringer, Mannheim, Germany) at 1 mg/ml diethanolamine as the substrate for 30 minutes at 25 °C and optical density was measured at 405 nm with a Titertek (Organon Teknika) reader.

Affinity of the MAb was determined in an ELISA system with rec gp120 (Microgenesys), using the method of Friguet et al. (1985). Data were analyzed with modifications for bivalency as described by Stevens (1987). Klotz-plots were used to determine the dissociation constants for the interaction of the anti-peptide antibodies with gp120. Conditions were chosen such as to allow the calculation of K_D with linear regression analysis according to Friguet et al. The equation used was $1/\nu = 1 + 1/a.K_D$. In this equation, a is the concentration free antigen and ν is the fraction of antibody bound.

PEPSCAN ANALYSIS

Pepscan analysis for mapping of the antibody binding site was performed as described earlier (Geysen et al., 1984) by testing reactivity of MAb with overlapping nonapeptides of the HIV-1 BH10 V3-domain. Nonapeptides were synthesized on polyethylene rods, which were used in an ELISA system. Sequences were obtained from Myers et al. (1990). Ascites of MAb IIIB-V3-34 was used at a 1:75,000 dilution and ascites of IIIB-V3-21 at 1:500. For MAb IIIB-V3-34, the contribution of individual amino acids within the epitope was analyzed by testing reactivity with a set of nonapeptides in which every amino acid was replaced by all possible others. Ascites was used at a 1:100,000 dilution. Parent sequence for this analysis was IQRGPGRAF.

FLOW CYTOMETRY

To assess if the ligand of the MAb is expressed on the surface of MOLT-3 cells and whether the MAb can be used for flow cytometry analysis of infected cells, cell surface staining was performed. MOLT-3 cells were washed three times in cold sorter buffer (PBS/2% FCS/0.1% NaN_3). 0.5 - 1 * 10^6 cells in sorter buffer were stained with appropriate dilutions of the primary antibody and were incubated for 30 min at 4 °C. After washing three times, a FITC-labeled secondary antibody was added, followed by the same incubation and washing procedure. 100 μl of 2% paraformaldehyde was added and cells were stored in the dark at 4 °C until use. Controls included non-infected MOLT-3 cells, incubation with the secondary antibody only, unstained cells and a positive control antibody reactive with gp120 on infected MOLT-3 cells. A Coulter Epics C flow cytometer was used.

IMMUNOCYTOCHEMISTRY

To determine if the ligand of MAb IIIB-V3-13 and IIIB-V3-34 was expressed on infected cells, drops of HIV-1 IIIB infected Sup-T1 cells (5 * 10^6/ml) were allowed to

dry on glass slides. Slides were fixed with acetone/methanol (1:1 v/v) for 10 minutes and viral activity was eradicated by subsequent incubation with ethanol (70%) for 30 min. After air-drying, slides were incubated with a titration series of the ascites of IIIB-V3-13 and IIIB-V3-34 in PBS containing 0.1% BSA (w/v) for 1 hour at 4°C. After rinsing three times with PBS, slides were incubated with Rabbit anti mouse IgG-HRP (horseradish peroxidase)(Dako) diluted 1:100 in PBS/BSA, supplemented with 1% (v/v) FCS, for 30 min at room temperature. HRP acitivity was revealed by incubating 10 min at room temperature with AEC (3-amino-9-ethylcarbazole) as described in detail elsewhere (Laman et al., 1990b). Controls included incubation with PBS/BSA only, omission of the primary antibody and staining of uninfected Sup-T1 cells.

HIV-1 NEUTRALIZATION ASSAYS

To test whether the different MAb and PAb have neutralizing capacity for IIIB and other HIV-1 strains, serum and ascites samples were tested in assays operational in different laboratories. Generally, coded samples including a negative control were used. SFI was performed as described previously (Gruters et al., 1987; Matthews et al., 1987) with minor modifications. Briefly, H9 cells (5 * 10^4/well) chronically infected with the HIV-1 isolates HTLV IIIB (Popovic et al., 1984), or the syncytium-inducing field isolates AMS 16 (Tersmette et al., 1988), ACH 105.33, ACH 479.7 (Tersmette et al., 1989) or ACH 320.5 (Groenink et al., 1991) were cocultivated in 96-well microtiter plates with high CD4-expressing Sup-T1 cells (1.5 * 10^5/well) in the presence of twofold serial dilutions of the different antibodies. After 16 h, syncytia were counted and 50% inhibition titers were determined, i.e. the highest dilution of a sample that caused 50% inhibition of syncytium formation in comparison to negative controls. A anti-HIV gammaglobulin preparation (HIVIG) derived from a plasma pool of seropositive donors (Prince et al., 1988) was used as a positive control.

In a cell-free virus neutralization assay, 100 μl of two-fold serial dilutions of the samples tested were mixed with 50 μl of diluted viral stock (viral infectivity 25-200 PFU/well). 100 μl medium containing 3 * 10^4 AA2 cells were added. Syncytia were counted after culturing for 5 days at 37°C in the presence of 5% CO_2 for 5 days. Titers are expressed as the highest dilution where 80% reduction of syncytia formation compared to the viral control is found.

RESULTS

GENERATION AND CHARACTERIZATION OF ANTI-V3 PEPTIDE ANTIBODIES

The amino acid composition analysis of the peptides that were synthesized was in accordance with the claimed sequence. The different peptides and their location in the V3-domain are indicated in table 1, together with the known V3-sequences of the field isolates used in SFI.

TABLE 1. V3-SEQUENCES, SYNTHETIC PEPTIDES AND ANTIBODY BINDING SITES

PEPTIDE	ISOLATE	RESIDUES	SEQUENCE OF V3-DOMAIN/PEPTIDE SHADED
SP 103	IIIB	298-315	SVEINCTRPNNNTRKSIRIQRGPGRAFVTIGKI.GNMRQAHC
SP 104	IIIB	314-328	CTRPNNNTRKSIRIQRGPGRAFVTIGKI.GNMRQAHC
SP 155	MN	310-324	CTRPNYNKRKRIHI . . GPGRAFYTTKNIIGTIRQ AHC
	AMS 16		CTRPNNNTRKGIHI . . GPGRAVYTTGRIIGDIRQAHC
	ACH 320.5		CTRPNNNTRKGIHI . . GPGRAFYAARKIIGDIRQAHC
	ACH 479.7		CTRPNNNTRKQIHI . . GPGRAFYTTRRIIGDIRQAHC
	ACH 105.33		ND

Full V3 sequences are shown and additional residues for SP 103. Numbering and sequences for IIIB are derived from Ratner et al. (1985); numbering and sequence for MN is according to Myers et al. (1990). Peptides used for immunization are shaded. A cystein residue was added to the N-terminus of SP 103 and the C-terminus of SP 104 and SP 155 for coupling purposes (not indicated in the table). The binding sites of MAb IIIB-V3-34 on SP 104 and of IIIB-V3-21 on SP 103 as determined by Pepscan analysis are underlined (see also figure 6 in chapter 8). Full stops are introduced in the sequences to obtain consensus sequences. ND: not determined.

The polyclonal serum responses in peripheral blood of immunized rabbits and mice and antibody production by hybridomas were screened by ELISA (results not shown), using unconjugated peptide, peptide-conjugates and rec gp120 as coating antigens. Depending on the ELISA titers of the animals, rabbits were bled to obtain PAb and mouse spleens were used for fusion to obtain MAb.
The PAb W0/07 reacted in ELISA with the immunogen SP 155 (MN) and also with SP 104 and rec gp120 (both IIIB; results not shown). The polyclonal anti SP 155 serum and the MAb obtained after immunization with SP 103 and SP 104 (table 1)

were further characterized as described below.

The affinity of MAb for rec gp120 was determined in an ELISA-system according to Friguet et al. (Friguet et al., 1985) and Stevens (1987). Results are shown in figure 1 and demonstrate that K_D of IIIB-V3-13 and IIIB-V3-34 is $6.8 * 10^{-11}$ and $1.6 * 10^{-10}$ M, respectively.

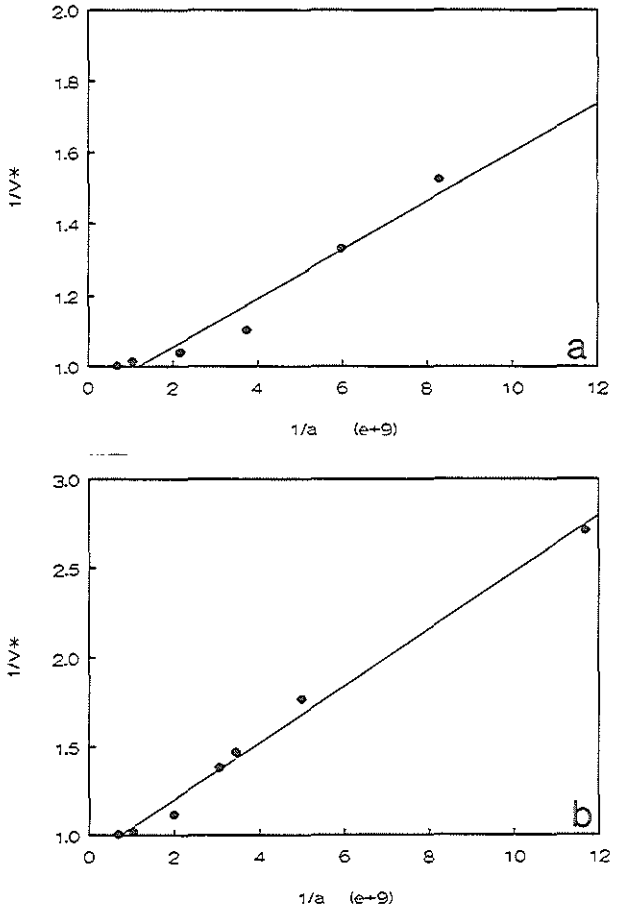

FIGURE 1. AFFINITY DETERMINATION OF MAB IIIB-V3-13 AND IIIB-V3-34 FOR RECOMBINANT GP120

Data are expressed as Klotz-plots. Linear regression analysis shows that for antibody IIIB-V3-13, $1/\nu = 0.91931 + 6.8 * 10^{-11} * 1/a$ with a correlation coefficient $R^2 = 0.985$. $K_D = 6.8 * 10^{-11}$ M (figure 1a). For antibody IIIB-V3-34, $1/\nu = 0.88460 + 1.5923 * 10^{-10} * 1/a$ with a correlation coefficient $R^2 = 0.994$. $K_D = 1.6 * 10^{-10}$ M (figure 1b).

2). The shortest reactive sequence of IIIB-V3-34 was found to be QRGP; best binding was found with the sequence IRIQRGPGR (figure 2a; see also table 1). IIIB-V3-13 has not been mapped, but may recognize the same site as it has been obtained from the same subcloning as IIIB-V3-34. IIIB-V3-21 mapped to the sequence INCTRPN (figure 2b; see also table 1).

To assess the contribution of individual amino acids to binding of MAb IIIB-V3-34, reactivity was tested with a set of peptides in which every residue of the sequences IQRGPGRAF was replaced by all possible others. Results (figure 3) show that the Q and GPG residues allow no or few replacements, while the two R residues allow more replacements, and the I, A and F are fully permissive for replacement.

EXPRESSION OF ANTIBODY-DEFINED EPITOPES ON HIV-1 IIIB-INFECTED CELLS

To assess expression and accessibility of the epitopes of the MAb on infected cells, IIIB-infected MOLT-3 cells were stained with IIIB-V3-13 for flow cytometry-analysis. Figure 3 shows that the SP 104 sequence of the V3-domain is highly expressed by infected cells, while non-infected cells show no staining with this antibody. A more detailed analysis of the accessibility to antibodies of V3-epitopes expressed on infected cells, on rec gp120, and on gp120 adsorbed to CD4-carrying cells will be presented elsewhere (Lewis et al., in preparation).

Surface expression of the SP 104-determinant on HIV-1 IIIB infected Sup-T1 cells was assessed by immunocytochemistry using MAb IIIB-V3-34. Figure 4 shows expression on infected Sup-T1 cells. As expression of the determinant is dependent on reproductive infection of an individual cell, non-infected or latently infected cells do not stain. Apparently, the conformational structure of the linear SP 104-determinant in the V3-domain is resistant to the denaturing effects of acetone/methanol fixation, followed by ethanol treatment. This determinant is also resistant to several other procedures for fixation and inactivation of HIV-1 (Laman et al., 1991c).

HIV-1 NEUTRALIZATION BY ANTI-V3 PEPTIDE ANTIBODIES

To assess if the different antibodies SP immunization have neutralizing capacity and if such neutralizing capacity is variant- or group-specific, neutralization studies were performed in two different assays, i.e. SFI and cell-free virus neutralization. The results of the SFI experiments are shown in table 2. High neutralizing activity, specific for the HIV-1 IIIB isolates was observed with

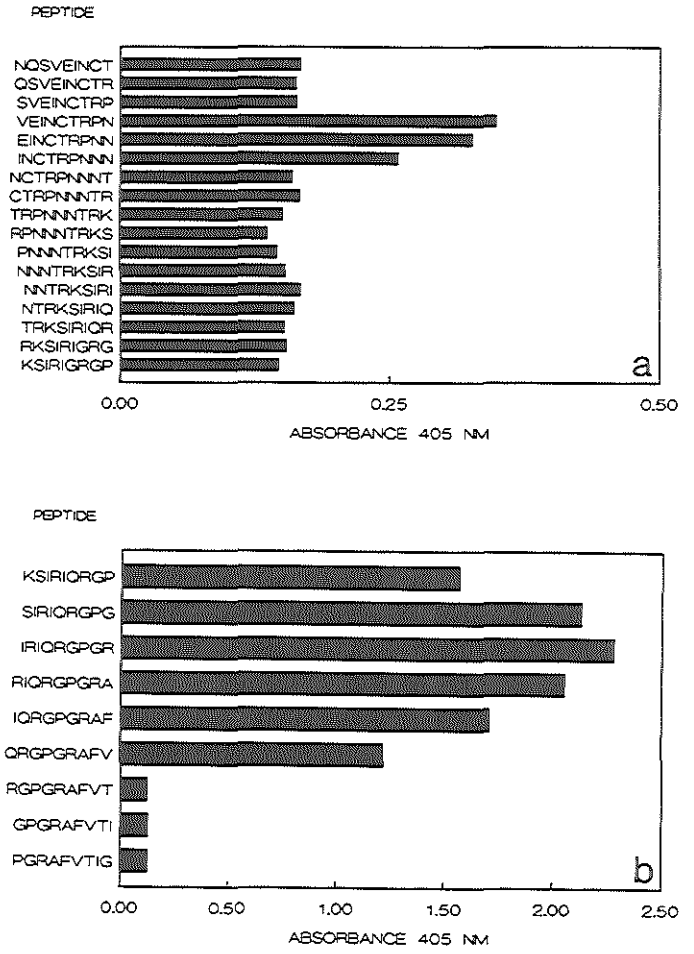

FIGURE 2. PEPSCAN ANALYSIS OF ANTIBODY BINDING SITES

Binding sites of two MAb were determined by Pepscan analysis, using overlapping nonapeptides. ELISA absorbancy values for binding of the antibody to the different peptides are shown.
Figure 2a. Mapping of IIIB-V3-21, raised with SP 103.
Figure 2b. Mapping of IIIB-V3-34, raised with SP 104.
Note different scales used for absorbance.

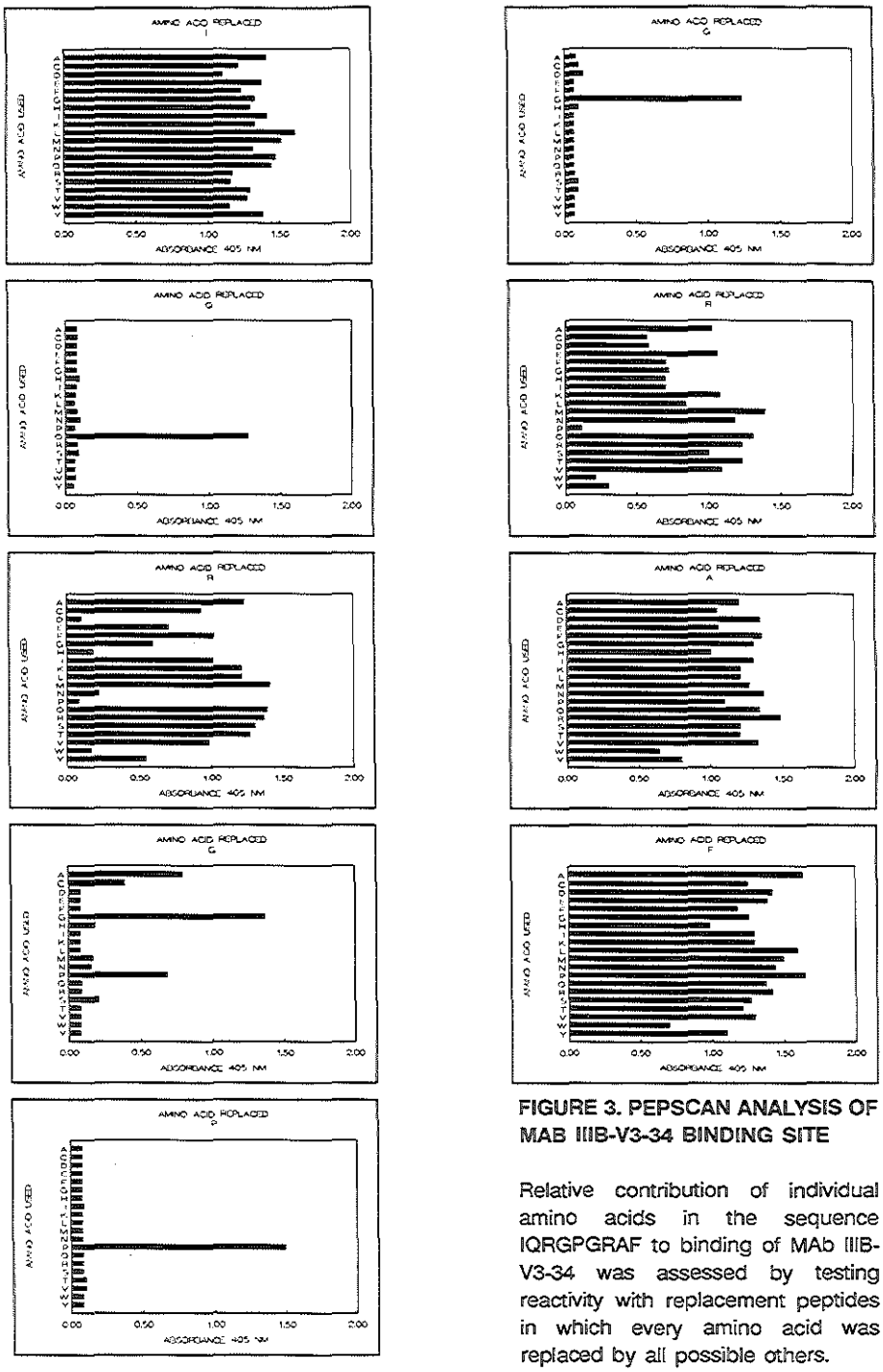

FIGURE 3. PEPSCAN ANALYSIS OF MAB IIIB-V3-34 BINDING SITE

Relative contribution of individual amino acids in the sequence IQRGPGRAF to binding of MAb IIIB-V3-34 was assessed by testing reactivity with replacement peptides in which every amino acid was replaced by all possible others.

the MAb IIIB-V3-13 and 34. These two MAb, however, did not significantly neutralize any of the four field isolates tested (no neutralization at the lowest dilution tested: 1:40).

PAb W0/07, raised against an MN V3-peptide (sample taken after first booster injection) exhibited low but broadly neutralizing activity against HIV-1 IIIB and four field isolates. The V3-sequences of three of these isolates were determined and revealed a high degree of homology with the MN sequence: AMS 16 (28/35 residues), ACH 320.5 (27/35 residues) and ACH 479.7 (30/35 residues)(table 1). Sequence homologies of field isolates with the MN V3-peptide SP 155 are: AMS 16 (12/15 residues), ACH 320.5 (11/15 residues) and ACH 479.7 (13/15 residues). The sample taken after the second booster had a somewhat lower titer in ELISA, and the neutralization titers were decreased compared to the first sample. However, significant neutralization of HIV-1 IIIB could still be demonstrated (data not shown).

TABLE 2. INHIBITION OF SYNCYTIUM FORMATION BY ANTI V3-PEPTIDE ANTIBODIES

ANTIBODY	HIV-1 ISOLATE				
	IIIB	ACH 105.33	AMS 16	ACH 320.5	ACH 479.7
A	<40	<40	<40	<40	<40
IIIB-V3-13	9000	<40	<40	<40	<40
IIIB-V3-34	3200	<40	<40	<40	<40
IIIB-V3-21	<40	<40	<40	40	<40
IIIB-V3-26	<40	<40	<40	<40	<40
W0/07	32	64	64	64	32
HIVIG	256	48	96	96	48

Patient isolates are T-cell tropic, syncytium inducing variants, obtained from patients of the Amsterdam cohort of homosexuals (ACH) or from patients from Amsterdam not involved in the cohort study (AMS). Antibody A is a negative control antibody. Data represent the reciprocal of 50% inhibition titers. Lowest dilution tested was 1:40 for MAb and 1:10 for PAb. Neutralization titers of IIIB-V3-13 and 34 are the average values of 2-4 experiments with duplicate observations. Titers for IIIB have been standardized by re-calculating the titer of HIVIG to 256. Titers of IIIB-V3-21, 26 and W0/07 are data from one experiment with duplicate observations.

Cell-free virus neutralization assays for the MAb gave essentially similar results: IIIB-V3-13 and 34 neutralized IIIB (HX10), but not MN. IIIB-V3-21 and 26 did not neutralize HX10 (table 3). Titers varied between assay-systems, which may be the result of numerous variables like cell lines, culture conditions, and viral stock used, and the percentage inhibition chosen to define neutralization. However, the trend was the same in all experiments, with IIIB-V3-13 neutralizing at slightly higher dilutions

than IIIB-V3-34. Neutralization by IIIB-V3-13, 34 and 21 has also been tested in assays operational in three other laboratories, yielding essentially identical results (not shown).

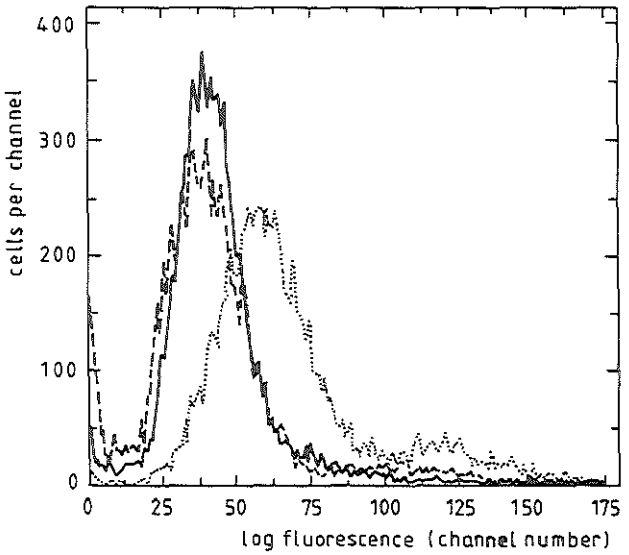

FIGURE 4. FLOW CYTOMETRY OF HIV-1 IIIB INFECTED MOLT-3 CELLS WITH MAB IIIB-V3-13

To assess surface expression of the epitope of IIIB-V3-13, MOLT-3 cells were analysed with flow cytometry. Results from a representative experiment are shown. Continuous line represents secondary antibody control on HIV-1 IIIB-infected cells. Dashed line represents MAb IIIB-V3-13 on uninfected MOLT-3 cells. Dotted line represents MAb IIIB-V3-13 on HIV-1 IIIB-infected MOLT-3 cells. Skewing of the dotted line to the right with respect to the control stainings demonstrates surface expression of the MAb IIIB-V3-13 defined epitope on the V3-domain.

TABLE 3. CELL-FREE VIRUS NEUTRALIZATION BY ANTI-V3 MAB USING AA2 CELLS

ANTIBODY	HIV-1 ISOLATE	
	IIIB	MN
NHS	<40	<40
1043-1	<100	<100
IIIB-V3-13	6400	<100
IIIB-V3-34	3200	<100

Neutralization of viral strains IIIB and MN by the MAb was tested in two separate experiments. Titers represent reciprocal of highest dilution giving 80% reduction in number of syncytia as compared to the viral control. Titers below 100 are considered to be non-neutralizing. 1:100 was the highest concentration of MAb tested. NHS is a negative human serum control of an HIV-1 seronegative person. 1043-1 is a negative control ascites of a clone that lost specificity during subcloning.

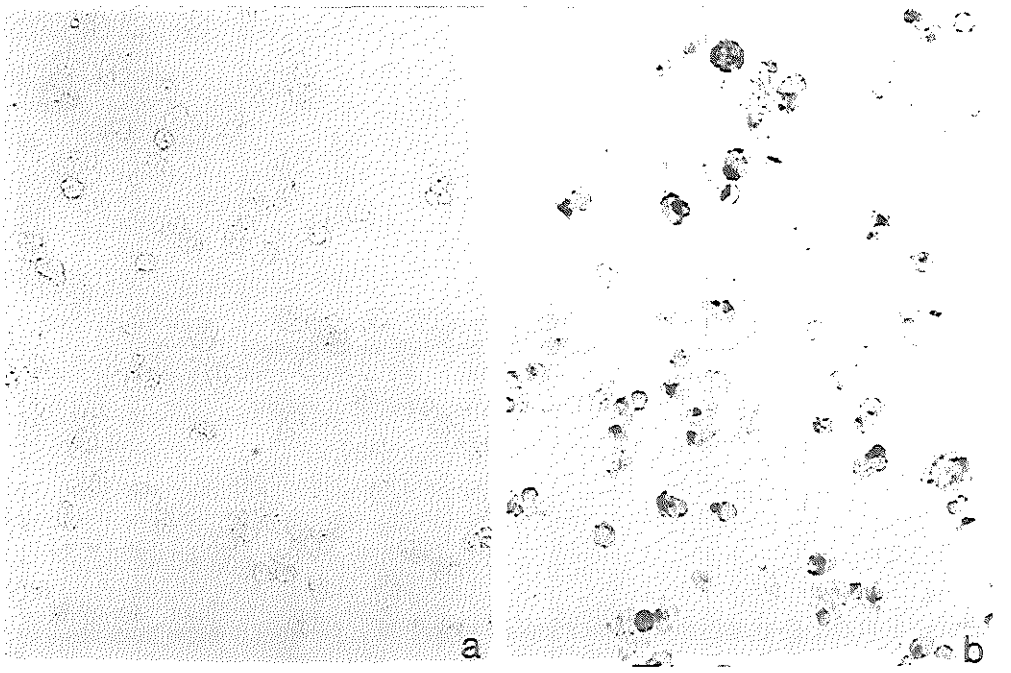

FIGURE 5. IMMUNOCYTOCHEMICAL DETECTION OF THE SP 104 DETERMINANT ON INFECTED CELLS

Panel a: control staining with omission of the primary antibody, showing no staining. Panel b: staining with ascites of MAb IIIB-V3-34, diluted 1:100,000, showing red staining of cells that express the SP 104 determinant.

DISCUSSION

This study reports the production and characterization of both group-specific neutralizing polyclonal antibodies and high-affinity variant-specific monoclonal HIV-neutralizing antibodies raised with synthetic peptides derived from the gp120 V3-domain.

HIV-1 vaccine strategies are often aiming for the induction of high titers of group-specific neutralizing antibodies, although it is not clear if a humoral anti-HIV-1 response can actually slow disease progression, prevent disease or prevent naturally occurring infection. Such strategies are facing a number of problems, two of which are relevant to the data presented here. First, hypervariability of HIV-1 complicates the design of immunogens evoking broad-spectrum neutralization. Second, it is not known how important the affinity of antibodies is for neutralizing activity.

Many studies have focused on the V3-domain of gp120, which evokes the major fraction of neutralizing antibodies during natural infection (Goudsmit et al., 1988; Rusche et al., 1988; Javaherian et al., 1989), as a candidate immunogen. Recent evidence indicates that sequence diversity of this domain is not as extensive as thought previously (LaRosa et al., 1990; Zwart et al., 1991) and that immunization with a single immunogen can give rise to group-specific antibody responses (Javaherian et al., 1990). Support for a role of anti-gp120 antibodies in protection against HIV-1 has been provided by Emini et al. (1990), who have shown that *in vitro* incubation with neutralizing antibody renders virus non-infectious for chimpanzees. In addition, candidate vaccines eliciting gp120 and especially V3-directed neutralizing antibody responses provided protection of chimpanzees against challenge with homologous virus after immunization with gp120 (Berman et al., 1990), or after immunization with a combination of inactivated virus, recombinant protein or V3-peptides (Girard et al., 1991).

These vaccine approaches are dependent on thorough analysis of the contribution of V3-sequences to antibody-mediated neutralization. In view of the variability of the V3-domain, it is crucial to assess the contribution of single amino acids to neutralization (e.g. Meloen et al., 1989; Langedijk et al., 1991) and to define cross-neutralization patterns of related but distinct sequences. Variant- and group-specific MAb and PAb are essential reagents in this respect. Several of these site-specific antibodies are described in this paper.

The MAb IIIB-V3-21 raised against the left-side base of the V3-domain did not neutralize HIV-1 infectivity. Apparently, this sequence is not critical to binding or fusion processes. This is in accordance with the hypothesis that the tip of the loop with the highly conserved GPGR sequence is critical for proper function. The flanking sequences can be highly variable without abolishing *in vivo* infectivity as evidenced by the field isolates obtained (Zwart et al., 1991).

The affinities of the MAb raised against the apex of the IIIb V3-domain are high (6.8 * 10^{-10} and 1.6 * 10^{-10} M; antibody affinities in general range from 10^{-5} M to 10^{-12} M), and they are comparable for both the reaction with rec gp120 and peptide (data not shown). Flow cytometry and immunocytochemistry showed that the epitope recognized by the MAb was accessible on infected cells and in addition on rec gp120 adsorped to CD4-positive cells. Thus, these antibodies may be of use in probing the mechanisms of attachment and penetration of HIV-1 (Lewis et al., manuscript in preparation).

In neutralization assays, neutralizing activity was only observed against the HIV-1 IIIB variant. This variant-specificity of MAb IIIB-V3-13 and 34 may result from the presence of the residues glutamin (Q) and arginin (R) in SP 104, which are only found in IIIB and not in more than 600 HIV-1 isolates from all over the world (Myers et al., 1990; Wain-Hobson et al., 1991). The possible role of these residues in binding of MAb IIIB-V3-34 has been investigated using a set of peptides with every amino acid replaced by all possible others. This analysis indeed showed that the presence of the glutamin residue is crucial to antibody binding. A replacement peptide analysis of a panel of other MAb binding to the V3-domain has been presented elsewhere (Langedijk et al., 1991). This study showed a correlation between antibody affinity and neutralizing activity.

The PAb W0/07 which has been raised with SP 155, exhibits broadly neutralizing activity against HIV-1 IIIB and four field isolates. This neutralizing activity is insensitive to substitutions of 2-4 amino acids in the field isolates when compared to the peptide sequence used for immunization.

These findings are in accordance with data on cross-neutralizing polyclonal antibodies, resulting from immunization with an MN peptide RP 142 YNKRKRIHIGPGRAFYTTKNIIG(C) (Javaherian et al., 1990). This peptide is 8 amino acids longer than our SP 155 (sequence underlined in RP 142). These data support the concept of peptide/subunit vaccines eliciting broad-spectrum neutralization.

In conclusion, we have shown that single-peptide immunization can result in group-specific HIV-1 neutralizing antibodies, and that high affinity variant-specific neutralizing monoclonal antibodies can be generated with gp120 V3-peptides. These antibodies are applicable in a variety of (bio-)assays and will contribute to the delineation of epitopes suitable for incorporation in candidate vaccines. In addition, application of these MAb in immunotoxin (e.g. Till et al., 1989) and heteroconjugates (Zarling et al., 1988) for *in vivo* elimination of infected cells can be envisaged, in view of the surface expression of the V3-domain *in vitro*.

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The references to this article are included in the References section following chapter 8.

CHAPTER 8

GENERAL DISCUSSION

8.1 INTRODUCTORY REMARKS

Detailed understanding of antibody formation in humans is desirable for rational vaccine development and elucidation of the histopathology and immunopathology of lymphoid organs occurring during diseases affecting or involving the lymphoid system. Although valuable information can be derived from serological studies and *in vitro* studies with peripheral blood lymphocytes from human subjects, such data do not reveal local events occurring in human lymphoid tissues, such as spleen and lymph nodes. Obvious ethical and practical considerations put restraints on experimental protocols to study humoral responses *in situ* in human lymphoid tissue. Such studies therefore have to rely on clinically obtained material. Data on *in situ* humoral respones have thus far only been derived from animal studies (cf. Van Rooijen et al., 1989b; Claassen et al., 1991b). However, species-specific differences in structure and function of lymphoid tissues exist (cf. Claassen et al., 1991b), complicating extrapolation of data obtained in animal studies to humans.

In this thesis we therefore chose to investigate antibody formation and follicular immune complexes *in situ* in an integrated approach, using both lymphoid tissues from experimentally immunized mice, and lymph node biopsies from humans from HIV-1 infected persons, providing a clinically relevant model. The papers included in this thesis show the advantages of the *in situ* approach for the unraveling of the sequence of events occurring during antibody formation and follicular immune complex localization. By using synthetic peptide-enzyme conjugates, techniques were developed to detect epitope specificity of antibody forming cells *in situ* that were subsequently used to visualize an antigen- and epitope-specific immune response directed against HIV-1 in human lymph nodes. To further unravel the immune response against HIV-1, antibodies against selected epitopes were produced and extensively characterized.

8.1 ANTIGEN SPECIFIC B-CELLS *in situ*

Several studies included in this thesis have addressed the development and localization of antigen/epitope-specific AFC *in situ*, using antigen-enzyme and peptide-enzyme conjugates for detection. Both in our animal studies and in human lymph node tissue we did not detect the cells that actually contain specific antibody in the follicles (Van Rooijen et al., 1989b; Laman et al., 1990b; 1991a). Follicular AFC were only found in rabbits, dependent on the immunization protocol (Van Rooijen et al., 1989b). In addition, T-cells containing cytokines presumably involved in regulation of specific B-cell responses (IL-2, IL-4 and IFN-γ) are also found outside the follicle (Van den Eertwegh et al., 1991a-c). This implies that the final stages of

conversion, from blast to actively antibody- or cytokine-producing cell, occur outside the follicles.

However, other groups have reported that the plasmacellular reaction occurs in the follicles (reviewed by MacLennan et al., 1991 and Szakal et al., 1989). Recently, Jacob et al. (1991) extensively studied the primary humoral response against (4-hydroxy-3-nitrophenyl)acetyl-carrier conjugates *in situ*. They found two distinct populations of AFC: one arising early (2 days) in the periphery of the PALS and a second appearing on day 8-10 in the germinal centers. No evidence was found that AFC migrate from the one population to the other. The germinal center AFC contain low quantities of immunoglobulin that can nevertheless be detected with antigen-enzyme conjugates, applying similar techniques as used in this thesis. Van Rooijen (1990b) has hypothesized that memory B-cells in the follicles may directly differentiate into AFC when two conditions are met: first, immune complexes with free antigenic determinants should be present in the follicle. Second, circulating antigen (i.e. free or in the form of soluble immune complexes) should be present. This situation may be mimicked experimentally by using adjuvants which slowly release the antigen or when a replicative pathogen is used.

In conclusion, the presence of AFC in the germinal center in the experimental setting may therefore be related to: 1) The interval between immunization and harvesting of the tissue for *in situ* analysis. 2) The use of an adjuvant which may change the primary character of the response (aluminum hydroxide in the study of Jacob et al., 1991). 3) The animal species under investigation. 4) The antigen/hapten used for immunization. A more trivial explanation would be that AFC in the follicles contain low affinity antibodies or low quantities of antibodies, which are unable to bind antigen-enzyme conjugates. This seems unlikely, however, because in that case, one would expect to find a continuum of weakly stained cells inside the follicle to brightly stained cells outside, when using antigen-enzyme conjugates.

In contrast to the extensive information on local antibody production in experimental animals after experimental immunization, little is known about naturally occurring *in situ* humoral responses in humans. Therefore, we aimed to compare specific antibody responses in lymphoid organs from humans suffering from progressive infectious disease with those from immunized experimental animals. In chapter 6, we have studied the *in situ* humoral response against proteins of HIV-1 in lymph node biopsies of HIV-1 infected individuals. Study of *in situ* antibody formation against HIV-1 is essential to determine how the tropism of the virus for CD4-carrying cells influences the humoral response and how histopathology and immunopathology are related to local antibody production. Spleens of HIV-1 infected persons are usually only available after the death of the patient. This material is not useful for immunocytochemistry as it shows poor morphology, lymphocyte depletion and autocytolysis. In contrast, frozen lymph node biopsies provide valuable material

for immunocytochemical analysis.

Two major conclusions with regard to AFC emerged from this study. First, the numbers of AFC found per square unit of tissue (up to about 300 per 40 mm^2) are very similar to those found after experimental immunization of animals, when lymphoid organs are analysed during the peak of the humoral immune response. Apparently, HIV-1 induces chronic stimulation of specific B-cells. Double staining with antigen-enzyme conjugates and isotype-specific antibodies may reveal whether a polyclonal activation of B-cells takes place, in addition to this HIV-1 specific response. Second, HIV-1 specific AFC were found solitary or as small clusters (up to 6-8 cells), which may represent clonal expansions, around the follicles in the interfollicular zone of the peripheral cortex. Only as an exception specific AFC could be found within the germinal center.

This localization of HIV-1 specific AFC in human lymph node biopsies is very similar to that of AFC found in the lymph nodes after experimental immunization of rodents with model antigens (reviewed by Van Rooijen, 1987b; Delemarre, 1990). The studies of rodent lymph nodes suggest that in T-cell dependent responses, B-cells meet the required T helper cells at the border of the T-cell area, i.e. mainly at the border between the extrafollicular zone of the peripheral cortex and the deep cortex. These T-cells may be activated by antigen presentation by interdigitating cells in the central parts of the deep cortex. Specific B-cells subsequently differentiate to AFC during migration along the periphery of the deep cortex to the medullary cords. AFC may leave the lymph node via medullary sinuses and efferent lymph vessels (Van Rooijen, 1987b). The localization of HIV-1 specific AFC in the human lymph node biopsies is fully accordant with this scheme. In conclusion, the localization and magnitude of the humoral response *in situ* is comparable for experimental animals and HIV-1 infected persons.

Both in the spleen and the lymph nodes, antigen presentation and antibody production are spatially separated, i.e. in the outer PALS and around the terminal arterioles in the spleen; in the intrafollicular areas and the medulla in the lymph node. Teleologically, it seems to make sense that the actual antibody production does not take place at the sites where antigen is presented. Here, as a rule, the pathogen has already been ingested, killed and degraded, followed by presentation of antigenic determinants. As the pathogen is rendered harmless, complexing of the non-viable antigens with specific antibody will not contribute to direct local protection. Such complexing may actually prevent efficient antigen presentation and hamper cell-cell interactions and migratory behaviour in these compartments. Secretion of antibodies around the terminal arterioles in the red pulp enables the specific antibodies to reach the systemic circulation almost instantaneously, to provide effective systemic protection. In addition, AFC can enter the bloodstream and localize to other parts of the body, such as the bone marrow. In this context it should be

realized, of course, that detection of intracellular immunoglobulin does not necessarily mean that the antibody is actually secreted in that specific location.

As discussed in chapter 2, it has become clear that antigen-specific antibody forming B-cells and cytokine-producing T-cells show localization/migration patterns that are independent of the type of antigen administered (i.e. T-cell dependent vs T-cell independent and/or soluble vs particulate). The compartmentalization of AFC and cytokine-producing T-cells implies that some compartments of the spleen (e.g. follicles, inner PALS) are not well suited for activated cells to secrete antibodies or to produce/receive activation signals like cytokines. This underscores the importance of the lymphoid microenvironment in facilitating or restricting interactions between cells and activation signals.

8.2 FOLLICULAR IMMUNE COMPLEXES

The data presented in chapter 4 obtained with size- and composition-defined antigen-enzyme conjugates, show that small immune complexes (i.e. consisting of one antibody and two antigen molecules) do not trap in splenic lymphoid follicles. This is in accordance with the requirement for complement activation to mediate trapping (Papamichail et al., 1975; Klaus and Humphrey, 1986), as interactions with multiple Fc-portions are necessary to activate complement and the trimolecular complexes only contain one Fc-portion. It also implies that Fc-receptors present in the follicle are not able to bind immune complexes containing a single immunoglobulin molecule. In chapter 4 it is shown that splenic macrophages and marginal zone B-cells are unlikely to be involved in transport of immune complexes, and that antigens can also be trapped in splenic follicles without involvement of specific antibodies.

This experimental evidence led us to investigate the role of follicular immune complexes *in vivo* in humans. To this end, we focused on *in situ* detection of HIV-1 immune complexes. Follicular trapping of HIV-1 proteins during a natural infection is of importance for two reasons. First, in view of the possible functions of immune complexes trapped in lymphoid follicles (see chapter 2), trapping of HIV-1 proteins could be pivotal to proper immune responsiveness against this virus, including the generation of B-cell memory. Second, the extensive and progressive histopathology of lymph nodes seen during HIV-1 infection (see chapter 3) may be related to the presence of HIV-1 antigens in the lymphoid follicles. We have hypothesized that HIV-1 proteins in lymphoid follicles may serve as a target for CD8+ cytolytic cells (Laman et al., 1989), which invade the follicles during HIV-1 infection, resulting in the observed severe damage to the network of follicular dendritic cells. Several other pathologic mechanisms initiated by HIV-1 and HIV-1 specific immune complexes in the follicles have been suggested (e.g. Rácz et al., 1989).

However, it is not clear whether HIV-1 is present in the follicles in the form of immune complexes. As a humoral response to HIV-1 occurs after infection (see chapter 3), follicular trapping of HIV-1 virions and/or proteins would be expected. HIV-1 virions and proteins have been demonstrated in the follicles by electronmicroscopy and by immunocytochemistry using specific antibodies, respectively (e.g. Rácz et al., 1989). Strong follicular localization of p24 is invariantly found, completely overlapping the specific staining for human FDC with the monoclonal antibody DRC-1 (e.g. Rácz et al., 1989). In contrast, despite numerous attempts by different groups, follicular localization of the transmembrane glycoprotein gp41 and the external membrane glycoprotein gp120 has not been demonstrated in lymph node biopsies using specific antibodies. Staining with HIV-1 antigen-enzyme conjugates (chapters 5 and 6) allows the detection of free antigen binding sites of antibodies present in trapped immune complexes. However, in our published series of five patients, and in an additional ten biopsies from other patients, we were unable to demonstrate p24-, gp160- or gp120-specific antibodies trapped in lymphoid follicles in the form of immune complexes.

There are several conceptual explanations for the presence of p24-antigen, and the apparent absence of gp120 and gp41 and gp120-, gp41- and p24-specific antibodies in the follicles. First, the epitopes recognized by the antibodies used for detection may be covered by host-produced antibodies, preventing binding of the detector antibody. If indeed the antibody production by the host covers all epitopes, then a situation of antibody excess would be achieved, leading to positive signals with antigen-enzyme conjugates. Negative results with both antibody staining and antigen-enzyme staining would only be found if there is complete equivalence of all antigen binding sites of host antibodies and their complementary epitopes. This possibility seems rather remote, however. As an example, figure 3k of chapter 2 shows p24-specific AFC in biopsy 1008/88 in which several hundreds of these bright-staining cells were found. This considerable number of AFC points to high-level production of anti-p24, presumably resulting in antibody excess. The same biopsy showed strong positivity for p24-antigen in the follicle, but had no free p24-specific antigen binding sites. This argues against p24 being present in the form of immune complexes. Second, all antigen binding sites of antibodies present in the immune complexes may be occupied by their complementary epitopes, preventing binding of antigen-enzyme conjugates. Third, gp120 and gp41 are in actuality not present in the follicles. This seems unlikely: as complete virions have been demonstrated in the follicle, gp120 and gp41 should be present, too. Fourth, p24 may be present in the follicles without being complexed by antibodies. p24-expression in the follicle may result from productive infection of FDC by HIV-1, presence of complete virions in the follicle, trapping of p24 without involvement of antibody or complement, or trapping of p24 through direct activation of complement.

Interactions of cell-free HIV-1, HIV-1 infected cells and HIV-1 membrane proteins with complement components have been described. C1q in human saliva binds to rec gp120 (Su and Boackle, 1991). C1q has been implicated in retention of immune complexes in lymphoid follicles of rat spleen (Maeda et al., 1988). Cell-free HIV-1 activates complement in the presence of specific antibody through the classical activation pathway, leading to formation of the C5b-9 membrane attack complex and subsequent virolysis (Spear et al., 1990). Complement alone does not lyse HIV-1 (cf. Spear et al., 1991). Sölder et al. (1989) have shown that purified virus and rec gp160 trigger the complement system through the classical pathway. Virus-infected cells trigger the alternative pathway of complement activation (Sölder et al., 1989). Yefenof et al. (1991) have shown that both HIV-1 and HIV-1 infected monocytic and T-cell lines can fix C3 fragments through the alternative pathway. Discrepancies between these studies may arise from different sources and concentrations of virus and human serum used. It is not clear whether free p24 (i.e. not complexed by specific antibody) can activate complement, leading to follicular trapping. Fifth, presence of immune complexes may depend on fluctuations in antibody titers against different HIV-1 proteins during progression of disease (Lange et al., 1986). This is possible, but in view of the long term retention of immune complexes (Tew et al., 1980) it is not expected to be an all-or-none phenomenon.

The apparent absence of trapped HIV-1 containing immune complexes may also be explained by technical limitations. First, quantities of trapped immune complexes may fall below detection levels by either antibodies or antigen-enzyme conjugates. Second, antibodies used for detection of gp120 and gp41 may not be sensitive enough. These possibilities can not be excluded with certainty. Third, host-produced antibodies could be of too low affinity to bind antigen-enzyme conjugates. This is unlikely as we are able to detect host-produced antibodies in specific AFC.

Further experiments are definitely required to determine if and how different HIV-1 antigens get trapped in lymphoid follicles, and what their role in immunopathology and B-cell responses is. Such experiments could include monitoring of repeated biopsies from patients or experimentally SIV/HIV-infected primates, culturing of purified FDC (Stahmer et al., 1991) to test trapping of immune complexes formed *in vitro*, and assessment of complement-activating capacity of p24.

8.3 FOLLICULAR DENDRITIC CELLS IN HIV-1 INFECTION

An important question related to HIV-1 specific immune complexes is the progressive destruction of the FDC-network. Different hypotheses have been put forward to explain this phenomenon, i.e. cytolysis by viral budding after productive infection of FDC, direct lytic effects of HIV-1 virions/proteins and cytolytic attack by

CD8+ T-cells. Although *in vitro* infection of purified FDC by HIV-1 has been demonstrated (Stahmer et al., 1991), the significance of this observation for FDC *in vivo* is unclear. In addition, in that study, no lytic activity of HIV-1 virions or proteins was reported. Strong support for the hypothesis that CD8+ T-cells in the follicles exert cytotoxic activity (chapter 6) comes from a recent study of Devergne et al. (1991), who demonstrated expression of the serine esterase B gene by *in situ* hybridization in lymphoid follicles of lymph node biopsies of PGL-patients. Serine esterase B is a protein present in cytoplasmic granules of cytotoxic T-cells. Parmentier et al. (1991b) could not find immunohistochemical evidence of granzyme B expression in CD8+ T-cells in lymphoid follicles. In view of the study of Devergne et al. (1991), this finding may be explained by technical caveats such as low sensitivity of the antibody used. In addition, CD8+ T-cell dependent follicle lysis has been described for the murine lymphocytic choriomeningitis virus (LCMV)(Odermatt et al., 1991). The conclusion of Parmentier et al. that cytotoxicity does not occur in the follicle is therefore premature and, in addition, disregards the existence of multiple mechanisms of cytotoxicity (Berke, 1991).

To develop therapeutic regimens delaying or preventing the dissolution of the FDC network, it is now important to ascertain whether cytotoxicity is the main mechanism involved and whether only serine esterase or also other cytotoxic mediators play a role. Immuno-electronmicroscopy and *in situ* hybridisation are useful techniques to approach these questions. In addition, the antigenic determinants for cytotoxicity have to be identified: are these HIV-1 epitopes expressed on infected cells, HIV-1 epitopes presented in the context of MHC class I molecules, or epitopes present in follicularly trapped immune complexes as proposed by Laman et al., 1989? Improved procedures for the isolation of human FDC (Stahmer et al., 1991) for *in vitro* studies allow co-culturing approaches to study susceptibility of FDC for different mechanisms of cytotoxicity and toxicity of HIV-1 virions and proteins. Finally, mechanisms leading to accumulation of CD8+ cells in the follicle, which is normally devoid of such cells, have to be elucidated.

It may be clear that the issue of prevention of damage to the FDC network is complex: CD8+ T-cells in the follicles may also serve to keep local HIV-1 replication in check (Walker et al., 1986; Martz and Howell, 1988). Strategies to prevent destruction of FDC by preventing influx of CD8+ cells may thus promote HIV-1 replication in the follicle.

8.4 HIV-1 SPECIFIC ANTIBODIES

HIV-1 specific antibodies were raised against synthetic peptides homologous to regions of the gp120 third variable domain, to investigate the involvement of this region in antibody-mediated neutralization (chapter 7). The synthetic peptides

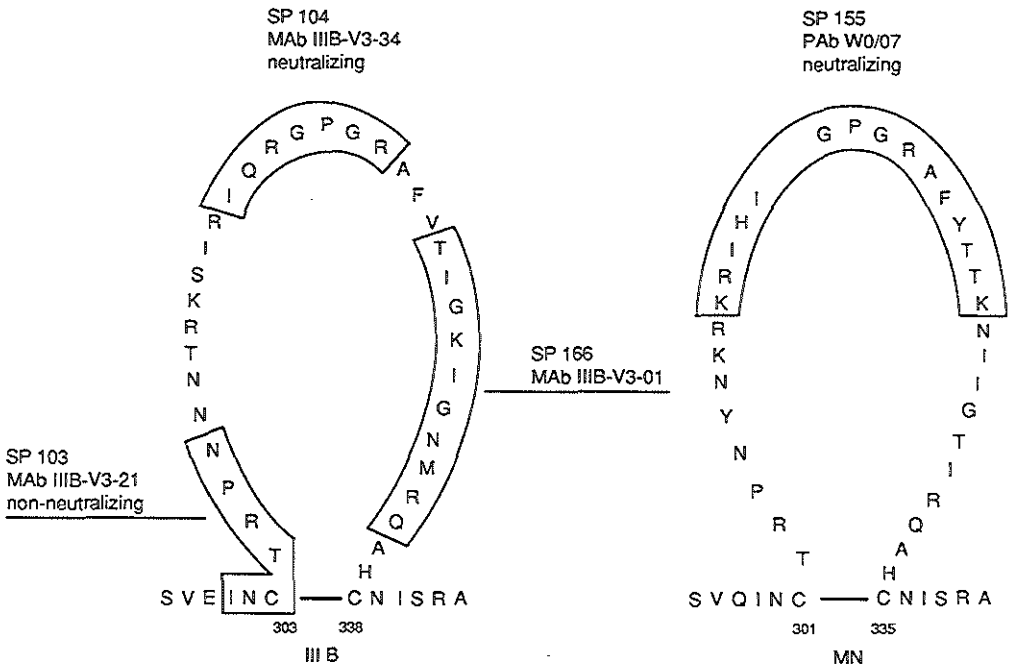

FIGURE 6. BINDING SITES OF HIV-1 SPECIFIC ANTIBODIES

As in figure 5 (see chapter 3), the amino acid sequence of the gp120 V3 domain of HIV-1 variants IIIB and MN are shown. Binding sites of different antibodies raised using synthetic peptides (chapter 7) are boxed. The numbers of the synthetic peptides (SP) used are indicated, as well as the code of the antibody and the possible neutralizing capacity. Polyclonal antibody W0/07 is broadly neutralizing. The monoclonal antibody MAb IIIB-V3-34 only neutralizes IIIB, which may be explained by the requirement of the Q (glutamin) and R (arginine) residues for binding of this antibody. MAb IIIB-V3-01 has not been described in this thesis, but is included here for reasons of completeness. Preliminary evidence indicates that this antibody does not neutralize HIV-1 IIIB, MN or RF (Dr. T. Matthews, pers. commun.).

and the binding sites of the different antibodies are indicated in figure 6.

These antibodies have been used to further unravel the role of the V3-domain in neutralization (reviewed by Goudsmit et al., 1991; Nara et al., 1991). Results confirm the importance of the GPGR-sequence in the apex of the loop, and the critical role of the amino acids Q and R (positions 317 and 318) in neutralization of

the HIV-1 IIIB isolate. Antibodies against the C-terminal part of the loop do not block infection or inhibit syncytium formation. The fact that neutralizing activity of rabbit polyclonal antibodies raised with peptide SP 155 is tolerant to 2-4 amino acid substitutions in the homologous sequence in the patient isolates (in a total of 15) lends support to the concept of peptide/subunit vaccination for the generation of broad-spectrum neutralizing antibodies.

The rabbit PAb W0/07 directed against the V3-domain of HIV-1 MN has also successfully been used to detect the presence of V3-sequences in brain specimens of AIDS-patients using immunocytochemistry. Expression of this V3-determinant was found at the cellular membrane of large macrophages, extracellularly and in the neuropilema (Budka, personal communication). In contrast, immunocytochemical staining of a series of ten lymph nodes of HIV-1 infected persons with this same PAb showed no positivity (Laman and Rácz, unpublished observations), indicating that this V3-determinant is probably not expressed *in situ* in the lymph nodes.

In conclusion, these antibodies generated with synthetic peptides display potent neutralizing activity mediated by binding to a functional viral determinant, showing that the peptides successfully mimick the conformation of the homologous sequence in the native protein. This biological activity pleads strongly against the contention that most anti-peptide antibodies or the linear sequences identified by them are devoid of any biological significance (Laver et al., 1990; Van Regenmortel, 1989). Although it may be true that most antibody epitopes are discontinuous, limiting the use of anti-peptide antibodies for epitope mapping, this does not permit the conclusion that functional sites in proteins are also necessarily discontinuous.

The data presented support the feasibility of using anti-peptide monoclonal and polyclonal antibodies to investigate the role of selected envelope sequences in cell tropism, attachment, penetration, and growth characteristics of distinct HIV-1 variants.

8.5 CONCLUDING PERSPECTIVE

As the lymphoid microenvironment restricts and dictates cell-cell interactions required for the humoral immune response, it is clear that *in vivo/in situ* investigations are needed to supplement data from *in vitro* experiments. The studies described in this thesis were designed to generate fundamental understanding of mechanisms involved in antibody formation and follicular immune complex localization, as well as to develop experimentally and clinically applicable techniques and reagents. The conceptual aspects with regard to antibody formation, follicular immune complexes and HIV-1 infection have been discussed in the respective chapters and in the preceding sections of this chapter.

Although we developed several methods and techniques with a specific

application in mind (e.g. studying HIV-1 specific AFC; see chapter 5), they are also useful tools in other research questions (reviewed by Claassen et al., in press, b). The techniques developed to detect epitope-specific antibodies in tissue sections, ELISA and ELISPOT using synthetic peptide-enzyme conjugates are readily applicable in experimental study of any other pathogen/antigen for which DNA or amino acid sequences have been determined.

The HIV-1 specific antibodies which were raised using synthetic peptides (chapter 7) are applied in the delineation of neutralization epitopes. In addition, the HIV-1 specific antibodies will be used for serotyping of patients, probing the role of different sequences in cell tropism of viral variants, studying the mechanisms of neutralization escape, and in technical applications such as viral protein purification and as a positive control in assays like ELISA and Western blotting. Further efforts in the production of site-specific anti HIV-1 antibodies will focus on sequences thought to be involved in cell tropism, growth characteristics and neutralization.

Several questions related to the humoral immune response both in experimental animals and humans require more experimentation. The relative contribution of different accessory cells to antigen presentation, the exact kinetics and migration routes of B-cells during different stages of differentiation (e.g. virgin, memory, plasmablast, plasmacell), the role of different cytokines in B-cell differentiation, and the relation between anatomical localization of B-cells and the genetic events controlling their function (such as isotype class switching, somatic mutation and affinity maturation) are all aspects needing better understanding. In combination with *in vivo* immunomodulation experiments, these *in situ* studies will provide relevant data on the way the immune system functions. This in turn will continue to lead to new rationales for the development of experimentally and clinically useful antibodies, vaccines, and therapy for auto-immune and immune deficiency diseases.

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SUMMARY

The aim of the experiments described in this thesis was to further elucidate the events occurring in the lymphoid microenvironment during specific antibody formation and follicular localization of immune complexes. Such knowledge is fundamentally important for understanding the humoral immune response. In addition, it may be practically applicable in the development of vaccines and in more efficient generation of antibodies in experimental animals for research, diagnostic and therapeutic purposes.

In chapter 1, the aim of the study and the *in situ* immunocytochemical approach used to address the research questions are described.

In chapter 2, the different cell-cell interactions occurring *in vivo* during the generation of antigen specific antibodies are reviewed. This chapter provides the essential background on the relation between the lymphoid microenvironment and the generation of the humoral immune response.

Chapter 3 provides a condensed background on HIV-1 infection, with special emphasis on the role of lymph nodes, HIV-1 specific antibody formation and vaccine development. This information serves to explain the selection of HIV-1 as a model pathogen of special interest to study antibody formation and follicular antigen localization.

In chapter 4, two aspects of follicular immune complex trapping were studied in experimental animals. We asked whether immune complexes composed of one antibody molecule and two antigen molecules localize in lymphoid follicles of mouse spleen. With a specially prepared monomeric HSA-HRP conjugate and HSA-specific antibodies we demonstrated that such complexes do not localize in the follicles. Next, we investigated whether murine splenic macrophages and marginal zone B-cells are involved in the transport of immune complexes to the follicles. *In vivo* elimination of these cells with a drug encapsulated in liposomes did not affect trapping of immune complexes, indicating that transport of immune complexes by these cells is not required.

To study antibody formation and follicular immune complex localization in relation to disease in humans and vaccine development, we chose HIV-1 as a pathogen. Chapter 5 describes the development of new immunocytochemical methods to study the presence and function of HIV-1 specific antibodies in tissues. We produced conjugates of synthetic peptides with detector enzymes, which allow the detection of epitope specificity of antibodies present in B-cells in tissue sections. In combination with isotype specific antibodies, immunocytochemical double staining can be performed to simultaneously demonstrate epitope specificity and isotype of the antibodies. Both recombinant gp120 and synthetic peptides could be used to induce specific antibody forming cells. We also tested a panel of fixation procedures

that inactivate HIV-1 for their suitability to reduce the biohazard risk of cryosections in immunocytochemistry. Several of these procedures were useful, as they did not interfere with staining of cell surface markers by monoclonal antibodies or with staining for specific antibodies using antigen-enzyme conjugates. Finally, we developed conjugates of synthetic peptides with different detector enzymes producing distinctly colored substrates. The availability of such conjugates significantly broadens the applicability of this technique.

The methods developed in chapter 5 were applied on cryosections of lymph node biopsies of HIV-1 infected persons (chapter 6). Both with recombinant antigen-enzyme conjugates and synthetic peptide-enzyme conjugates specific antibody forming cells could be detected. This was the first visualization of an antigen specific immune response in human tissue. We also proposed a hypothesis to explain the damage to follicular dendritic cells occurring in HIV-1 infected persons. The antigens present in the immune complexes on these cells may be the target for attack by cytolytic cells.

Chapter 7 describes the generation of HIV-1 specific monoclonal and polyclonal antibodies in experimental animals by using synthetic peptides homologous to regions of the gp120 V3-domain. Some of these antibodies were shown to neutralize one or more HIV-1 strains *in vitro* and can therefore be used for the delineation of neutralization epitopes. In addition, these data provide information relevant to development of peptide vaccines. Other applications of these antibodies are the characterization of viral variants and studies of viral expression and gp120 folding.

In chapter 8, the general discussion, the main points emerging from the different studies are discussed concisely with respect to antibody formation, follicular immune complexes and vaccine development. It is argued that antibody formation in human lymph nodes during HIV-1 infection closely resembles the humoral response in experimental animals after immunization in terms of numbers and location of specific B-cells. As specific B-cells are seldomly found inside the follicles it seems that this is not the compartment where the plasmacellular reaction takes place. With regard to follicular immune complex trapping, the data presented question the concept of cell-mediated transport of immune complexes. In HIV-1 infected humans, no evidence was found for trapping of HIV-1 containing immune complexes. Possible explanations for these findings are discussed. The data obtained with HIV-1 specific antibodies demonstrate that synthetic peptides can be used to generate humoral responses that neutralize different HIV-1 strains. Finally, the general applicability of the new immunocytochemical methods developed in immunology and cellular biology is discussed.

SAMENVATTING

De experimenten beschreven in dit proefschrift hebben tot doel een beter begrip te krijgen van de processen die plaatsvinden in het lymfoïde micromilieu bij vorming van antigeenspecifieke antilichamen, en bij de lokalisatie en functie van antigeen-antilichaam immuuncomplexen in de lymfoide follikels (folliculaire trapping). Inzicht in deze mechanismen is belangrijk voor het doorgronden van de humorale immuunrespons, en kan daarom bijdragen aan de ontwikkeling en verbetering van vaccins en aan het doelmatiger opwekken van antilichamen in proefdieren voor experimentele, diagnostische en therapeutische toepassingen.

Het doel van de studies en de *in situ* immunocytochemische benadering die wordt gebruikt worden beschreven in hoofdstuk 1.

In hoofdstuk 2 worden de verschillende cel-cel interacties besproken die ten grondslag liggen aan de produktie van specifieke antilichamen. Dit hoofdstuk legt de nadruk op het verband tussen de organisatie van het lymfoïde micromilieu en de humorale immuunrespons.

Hoofdstuk 3 verschaft een beknopt overzicht van HIV-1 (human immunodeficiency virus type 1) infectie, waarbij de nadruk ligt op de lymfeklieren, HIV-1 specifieke antilichaamvorming en vaccinontwikkeling. Deze informatie dient tevens om de keuze van HIV-1 als een model pathogeen voor het bestuderen van antilichaam formatie en folliculaire lokalisatie van antigenen toe te lichten.

In Hoofdstuk 4 werden twee aspecten van folliculaire lokalisatie van immuuncomplexen bestudeerd in de muis. We onderzochten of immuuncomplexen bestaand uit een enkel antilichaam en twee antigeen moleculen kunnen lokaliseren in follikels van de milt. Met een speciaal geconstrueerd monomeer HSA-HRP conjugaat en HSA-specifieke antilichamen werd aangetoond dat dergelijke complexen daar niet lokaliseren. Vervolgens werd onderzocht of milt macrofagen en marginale zone B-cellen immuuncomplexen transporteren naar de follikels. *In vivo* eliminatie van deze cellen met een drug ingesloten in liposomen beïnvloedde lokalisatie van de complexen niet, hetgeen aangeeft dat de aanwezigheid van deze cellen niet noodzakelijk is voor folliculaire lokalisatie.

Om antilichaamvorming en folliculaire lokalisatie van immuuncomplexen te bestuderen in de mens en in relatie tot vaccinontwikkeling, werd het retrovirus HIV-1 als pathogeen gekozen. Hoofdstuk 5 beschrijft de ontwikkeling van nieuwe immunocytochemische methoden om HIV-1 specifieke antilichamen in weefsels te kunnen bestuderen. We produceerden conjugaten van synthetische peptiden met detector enzymen, waarmee de epitoopspecificiteit van antilichamen aanwezig in B-cellen in vriescoupes kon worden aangetoond. Door een dubbelkleuring uit te voeren met deze conjugaten en met isotype specifieke antilichamen, kon tegelijkertijd de epitoop specificiteit en het isotype van de antilichamen in de coupe worden

bepaald. Zowel met recombinant gp120 als met synthetische peptiden kunnen specifieke antilichaamvormende cellen worden opgewekt. Om het infectiegevaar van werken met HIV-1 geïnfecteerde vriescoupes te reduceren werd een aantal fixatieprocedures die tevens HIV-1 kunnen inactiveren getest. Enkele van deze methoden waren bruikbaar daar ze niet interfereerden met kleuring van membraaneiwitten d.m.v. monoclonale antilichamen of van antilichamen d.m.v. antigeen-enzym conjugaten. Tenslotte ontwikkelden we conjugaten van synthetische peptiden met verschillende detector enzymen die verschillend gekleurde produkten geven. Zulke conjugaten breiden de toepassingsmogelijkheden van deze detectietechniek aanzienlijk uit.

De methoden die werden ontwikkeld in hoofdstuk 5 werden toegepast op vriescoupes van lymfeklieren van HIV-1 geïnfecteerde personen (hoofdstuk 6). Zowel met recombinant eiwit-enzym conjugaten als met synthetisch peptide-enzym conjugaten konden antigeen-specifieke antilichaamvormende cellen worden aangetoond. Dit was de eerste maal dat een antigeen-specifieke immuunrespons zichtbaar gemaakt werd in humaan weefsel. We formuleerden daarnaast een hypothese om de afbraak van follicularie dendritische cellen gedurende HIV-1 infectie te verklaren: antigenen aanwezig in de immuuncomplexen op het oppervlak van deze cellen vormen mogelijk het doelwit voor cytolytische cellen.

Hoofdstuk 7 beschrijft de produktie van HIV-1 specifieke monoclonale en polyclonale antilichamen door het gebruik van synthetische peptiden van het V3-domein van gp120. Sommige van deze antilichamen neutraliseren een of meer HIV-1 varianten *in vitro* en kunnen daarom gebruikt worden voor het definiëren van neutralisatie epitopen. Deze antilichamen kunnen ook gebruikt worden voor de karakterisering van virale varianten en studies naar expressie en structuur van gp120. Bovendien zijn deze gegevens van belang voor de ontwikkeling van peptide-vaccins.

In de algemene discussie, hoofdstuk 8, worden de belangrijkste punten die uit de diverse studies naar voren komen kort besproken in het licht van antilichaamvorming, follicularie immuuncomplexen en vaccinontwikkeling. Antilichaamvorming tijdens HIV-1 infectie lijkt sterk op de humorale respons in proefdieren na immunisatie, in termen van aantallen en lokatie van specifieke B-cellen. Omdat specifieke B-cellen zelden in de follikels worden gevonden, lijkt de follikel niet de plaats te zijn waar de plasmacellulaire reactie plaatsvindt. De gegevens met betrekking tot immuuncomplexen wijzen erop dat transport door cellen niet noodzakelijk is voor follicularie lokalisatie. In HIV-1 geïnfecteerde personen konden geen aanwijzingen worden gevonden voor follicularie lokalisatie van HIV-1 bevattende immuuncomplexen. Een aantal verklaringen hiervoor wordt besproken. De gegevens verkregen met HIV-1 specifieke antilichamen tonen aan dat synthetische peptiden gebruikt kunnen worden om humorale responsen op te

wekken die verschillende HIV-1 varianten kunnen neutraliseren. Tenslotte wordt de algemene toepasbaarheid van de nieuw ontwikkelde immunocytochemische methoden in andere vraagstellingen besproken.

SAMENVATTING VOOR DE NIET-IMMUNOLOOG

IMMUNITEIT

Levende organismen worden voortdurend bedreigd door infecties met virussen, bacteriën, schimmels en parasieten. Als antwoord op die dreiging beschikken levende organismen over tal van beschermingsmechanismen. Die mechanismen lopen uiteen van een simpele afscherming van de buitenwereld door middel van een barrière (bijvoorbeeld de huid) via bacterie-dodende enzymen in lichaamsvochten tot het complex georganiseerde lymfoïde systeem. Het lymfoïde systeem (o.a. beenmerg, thymus, milt, lymfeklieren en witte bloedcellen) is verantwoordelijk voor het tot standkomen van immuniteit, het immuun zijn voor een bepaalde ziekteverwekker.

De twee kenmerken van verworven immuniteit zijn specificiteit en geheugen. Nadat een persoon bijvoorbeeld eenmaal mazelen heeft gehad (of ervoor is ingeënt), wordt hij na een volgend contact met het virus niet meer ziek (de bescherming heeft geheugen). De bescherming geldt in dit geval echter alleen mazelen en niet een andere ziekteverwekker (de bescherming is specifiek).

Deze immuniteit berust op twee verschillende typen witte bloedcellen (lymfocyten), namelijk T-cellen en B-cellen. We onderscheiden twee soorten T-cellen, nl. T-cellen die lichaamseigen cellen die afwijkende trekken vertonen onder invloed van een ziekteverwekker kunnen doden, en T-cellen die de activiteit van andere T-cellen en van B-cellen stimuleren (T-helper cellen). B-cellen kunnen antilichamen (eiwitmoleculen) maken die uiterst specifiek een klein onderdeel (epitoop) van een ziekteverwekker (antigeen) herkennen. Deze antilichamen kunnen het antigeen binden en op verschillende manieren onschadelijk helpen maken. Als een antilichaam bindt aan een antigeen, noemen we dit antigeen-antilichaam complex een immuuncomplex. Immuuncomplexen worden voor een gedeelte vastgehouden in speciale gebieden van de milt en de lymfeklieren, namelijk de kiemcentra (follikels). In deze gebieden liggen veel B-cellen en er zijn aanwijzingen dat immuuncomplexen te maken hebben met het tot stand komen van het immunologisch geheugen van de antilichaamvorming.

DOEL VAN DE STUDIE

Dit proefschrift gaat over antilichaamvorming en de immuuncomplexen die ontstaan bij deze antilichaamrespons. Er is onderzoek gedaan naar de mechanismen die betrokken zijn bij de lokalisatie van immuuncomplexen in de lymfoïde follikels. Er werden methoden ontwikkeld om in dunne weefselplakjes (coupes) te kunnen vaststellen tegen welke epitoop van een antigeen de antilichamen in B-cellen gericht

zijn. Deze methoden werden toegepast op lymfeklieren van personen die besmet zijn met het humaan immunodeficientie virus type 1 (HIV-1), het virus dat AIDS veroorzaakt. Zo kon vastgesteld worden om vast te stellen of, waar en hoeveel B-cellen er aanwezig waren die antilichamen maken die het virus binden. Tenslotte werden er in proefdieren antilichamen gemaakt die aan epitopen van HIV-1 binden en de infectiviteit van het virus kunnen neutraliseren.

BELANG VAN DE VRAAGSTELLING

Wat voor belang hebben de vragen van dit onderzoek? In de eerste plaats is het fundamenteel-wetenschappelijk gezien interessant om te weten hoe de antilichaamrespons plaatsvindt en welke processen een rol spelen bij de folliculaire lokalisatie van immuuncomplexen in proefdieren en in de mens. In de tweede plaats kan dergelijke kennis van belang zijn voor het ontwikkelen en verbeteren van vaccins, en voor het efficiënter opwekken in proefdieren van antilichamen die toepasbaar zijn in onderzoek, diagnostiek en therapie. De nieuwe immunocytochemische methoden die we ontwikkeld hebben kunnen toegepast worden in tal van andere experimentele systemen.

HIV-1 werd gekozen om bij te dragen aan het begrip van de antilichaamvorming tegen dit virus, de afwijkingen die het veroorzaakt in de lymfeklieren, en om epitoop-specifieke antilichamen te produceren die een directe experimentele toepassing in vaccinontwikkeling vinden.

IMMUNOCYTOCHEMIE EN SYNTHETISCHE PEPTIDEN

Veel immunologisch onderzoek wordt uitgevoerd met cellen die geïsoleerd worden uit het bloed of door lymfoïde organen stuk te knippen. De cellen worden vervolgens in een steriele kweek gebracht: de functie van cellen wordt zo *in vitro* (in plastic flesjes en buisjes) onderzocht. Lymfoïde organen zijn echter sterk georganiseerde structuren met verschillende compartimenten: de talrijke verschillende celtypen worden alleen op karakteristieke lokaties gevonden. Het is aannemelijk dat deze strikte organisatie zijn weerslag vindt in de activiteit van de verschillende celtypen. *In vitro* onderzoek kan met die complexe organisatie echter per definitie geen rekening houden.

Omdat wij juist de rol van de organisatie belangrijk achten en willen analyseren zijn de vraagstellingen van dit proefschrift veelal benaderd door van diepgevroren lymfoïde organen uiterst dunne plakjes te snijden (coupes). In die coupes werden antilichamen en cellen zichtbaar gemaakt met kleurreactie (immunocytochemische kleuringen) en beoordeeld met de lichtmicroscoop. Omdat op deze manier cellen in hun natuurlijke omgeving worden bestudeerd, wordt dit

de *in situ* (ter plaatse) benadering genoemd. Zo kunnen de verbanden tussen anatomische lokalisatie van antigeen, geïnfecteerde cellen, specifieke antilichaamvormende cellen en andere celtypen onderzocht worden.

Bij immunocytochemie wordt gebruik gemaakt van antilichamen die uiterst specifiek binden aan bepaalde structuren (hun epitoop). Door de antilichamen te koppelen (conjugeren) aan een detectorenzym dat een kleurreactie kan geven, kunnen epitopen en dus de antigenen waarvan ze deel uitmaken in de coupe worden aangetoond. Een andere immunocytochemische techniek is het aantonen van specifieke antilichamen in de coupe door juist het antigeen te koppelen met een detectorenzym. Als een dergelijk conjugaat op een coupe wordt gebracht, binden de specifieke antilichamen gevormd door de B-cellen aan de corresponderende epitoop van het antigeen-enzym conjugaat. In deze studies is gebruik gemaakt van intacte eiwitten als antigenen. Bovendien werden epitopen van HIV-1 antigenen nagebootst door op synthetische wijze aminozuren (de afzonderlijke bouwstenen van eiwitten) aan elkaar te koppelen tot peptiden. Door deze synthetische peptiden te koppelen met verschillende detectorenzymen werden peptide-enzymconjugaten verkregen. Met deze conjugaten kan de epitoop-specificiteit van B-cellen en folliculaire immuuncomplexen in coupes worden aangetoond. Synthetische peptiden werden ook gebruikt voor het opwekken van HIV-1 specifieke antilichamen in proefdieren. De principes van enkele immunocytochemische technieken worden uitgelegd in figuur 1 van hoofdstuk 1.

HIV-1

De studies van dit proefschrift zijn grotendeels gericht op HIV-1. Hoewel er over de structuur en biologie van het virus zelf inmiddels bijzonder veel bekend is, blijft het onduidelijk hoe de ziekte tot stand komt. HIV-1 infectie brengt dramatische veranderingen in de structuur en organisatie van de lymfoïde organen met zich mee. Ook hiervoor is nog geen afdoende verklaring. De veranderingen in de lymfoïde organen liggen mogelijk ten grondslag aan de verminderde functie van T- en B-cellen, die zich uit als een immunodeficiëntie: dit is het onvermogen om adequaat te reageren tegen allerlei infecties. Met de immunocytochemische technieken die in dit onderzoek gebruikt werden, kan informatie worden verkregen over specifieke B-cel reactiviteit en over de rol van folliculaire immuuncomplexen tijdens HIV-1 infectie. De HIV-1 specifieke antilichamen die zijn opgewekt zijn nuttige hulpmiddelen bij het ontwikkelen van vaccins, en kunnen tal van andere toepassingen vinden in bijvoorbeeld karakterisering en zuivering van virusstammen en eventueel in therapeutische benaderingen.

INHOUD VAN HET PROEFSCHRIFT

In hoofdstuk 1 wordt het doel van de studies in dit proefschrift besproken en wordt uitgelegd welke experimentele benadering er wordt gebruikt.

In hoofdstuk 2 wordt een overzicht gegeven van de interacties die optreden tussen verschillende celtypen in het lymfoïde micromilieu tijdens de antilichaamrespons, en van de folliculaire lokalisatie van immuuncomplexen.
Hoofdstuk 3 geeft beknopte informatie over infectie met HIV-1 en de immuunrespons tegen HIV-1. Daarbij ligt de nadruk op de rol van de lymfeklieren, antilichaamvorming en vaccinontwikkeling.

In hoofdstuk 4 wordt beschreven hoe in muizen is vastgesteld dat zeer kleine immuuncomplexen niet in de follikels terechtkomen. Daarnaast werd gevonden dat bepaalde vreetcellen (macrofagen) in de milt niet betrokken zijn bij transport van immuuncomplexen.

Hoofdstuk 5 beschrijft de ontwikkeling van nieuwe methoden om in menselijk materiaal de antilichaamrespons tegen HIV-1 zichtbaar te maken. Daarvoor werden peptiden die stukjes van het virus nabootsen aan verschillende typen enzymen gekoppeld die aanleiding geven tot verschillende kleuren. Er werd een methode ontwikkeld om tegelijkertijd het type antilichaam (er bestaan vijf typen) en de specificiteit van antilichamen in coupes aan te tonen. Bovendien werden methoden getest die HIV-1 inactiveren, zonder de antigenen en antilichamen in het weefsel te beschadigen.

De methoden die zijn beschreven in hoofdstuk 5 werden toegepast op lymfeklieren van HIV-1 geïnfecteerde personen (hoofdstuk 6). Met antigeen-enzym conjugaten en met synthetisch peptide-enzym conjugaten konden specifieke B-cellen worden aangetoond. Dit verschaft informatie over de antilichaamrespons zoals die in humane lymfeklieren plaatsvindt. Tevens is een hypothese opgesteld om de schade die optreedt aan een bepaald celtype dat van groot belang is voor de antilichaamrespons in de lymfeklier te verklaren.

Met behulp van synthetische peptiden van HIV-1 werden in proefdieren antilichamen opgewekt die de werking van HIV-1 *in vitro* belemmeren (hoofdstuk 7). Zulke antilichamen vormen een nuttig gereedschap bij bestuderen van HIV-1 varianten en het ontwikkelen van vaccins.

In de algemene discussie worden de belangrijkste punten die uit de verschillende studies naar voren komen besproken in het licht van antilichaamvorming, folliculaire immuuncomplexen en vaccinontwikkeling. Bovendien wordt aangegeven hoe de verschillende methoden die ontwikkeld zijn ook kunnen worden toegepast in andere studies.

CURRICULUM VITAE

Jon Daniël Laman werd op 25 maart 1964 geboren te Waalre. In 1982 werd het VWO-diploma behaald op het Haarlemmermeer Lyceum te Badhoevedorp. In 1987 werd het doctoraaldiploma Medische Biologie behaald aan de Faculteit der Biologie van de Vrije Universiteit te Amsterdam (VUA). De onderzoekstages voor dit doctoraaldiploma werden gedaan op de afdeling Histologie van de Faculteit der Geneeskunde van de VUA (hoofd Prof.dr. T. Sminia, begeleider dr. G. Kraal) en de afdeling Immunobiologie van het Rijksinstituut voor Volksgezondheid en Milieuhygiene (RIVM) te Bilthoven (hoofd Prof.dr. A.D.M.E. Osterhaus, begeleider dr. A.G.C.M. UytdeHaag). Van april 1987 tot juli 1988 was de auteur als wetenschappelijk onderzoeker verbonden aan de afdeling Histologie van de Faculteit der Geneeskunde van de VUA (begeleider dr. N. Van Rooijen). Sinds juli 1988 is Jon Laman als wetenschappelijk onderzoeker verbonden aan de Sectie Immunologie en Medische Microbiologie van het Medisch Biologisch Laboratorium TNO (MBL-TNO) te Rijswijk (hoofd dr. E. Claassen).

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* not incorporated in this thesis

@ containing original colour photo micrographs which have been reproduced in black and white in this thesis

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