

IMMUNITY AGAINST THE MOUSE MAMMARY TUMOUR VIRUS

Immunologic Events during Tumour Growth and Studies on
Vaccination in Mice

PROEFSCHRIFT

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"My life is spent in one long effort
to escape from the commonplaces of
existence"

SHERLOCK HOLMES

ERRATA

Corrections are underlined.

- p. 12: **See text (page 13).
- p. 13, line 4-5: mouse strains.
- p. 14, line 2:that carries the endogenous
MTV-P, the DNA of tumour cells and normal
tissues was found to contain.....
- p. 22, line 16:.....the BALB/c, C3Hf, BALB/cfC3H....
- p. 26, line 7:.....see II, 8).....
- p.120, legend figure 15B:...from vaccinated mice;
.....:spleencells from normal mice.
- p.133, line 36;virus coded TSSA('s)may be ex-
pressed.....
- p.142, line 22:directe en de indirecte LAI-test
uitgebreid.....
- p.163, line 12-13:beschreven het experimentele
werk dat hier.....

On the cover are presented an electron microscopic photograph of the mammary tumour virus and mice bearing transplanted mammary tumours.

The drawings are made by Marja Proper.

CONTENTS

Chapter I	INTRODUCTION	9
	1.1. Characteristics of the mouse mammary tumour virus	9
	1.2. MTV expression and mammary tumour development in different mouse strains	13
	1.3. Immunity to MTV in healthy animals	15
	1.4. Immunity to MTV in tumour-bearing animals	17
	1.5. Vaccination experiments	19
	1.6. Rationale for the investigation: relation of the mouse model to human breast cancer	22
Chapter II	TECHNICAL APPROACH	25
	2.1. Animals	25
	2.2. Virus purification	26
	2.3. The direct leukocyte adherence inhibition (LAI) assay	27
	2.4. The indirect LAI assay	31
	2.5. Leukocyte stimulation (LS) test	37
	2.6. Separation and testing of spleen cell subpopulations	42
	2.7. Detection of MTV and of mouse anti-MTV-antibodies.	43
	2.8. Vaccination experiments	46
Chapter III	MTV-DIRECTED IMMUNITY IN MICE DURING THE GROWTH OF SPONTANEOUS TUMOURS	51
	3.1. Membrane immunofluorescence	51
	3.2. Direct LAI assay	55
	3.3. LS test	57
	3.4. Discussion	60
CHAPTER IV	THE ROLE OF SPLEEN CELL SUBPOPULATIONS IN MTV-DIRECTED CELLULAR IMMUNITY	63
	4.1. Mechanisms of MTV-induced LAIF-production	63
	4.2. MTV-specific proliferating cells in the LS assay	69
	4.3. Discussion	73

Chapter V	SUPPRESSIVE FACTORS INTERFERING WITH CELLULAR IMMUNE RESPONSES TO MTV IN TUMOUR BEARING MICE	77	
	5.1	Suppressor cells with adherent properties	77
	5.2.	Suppressive factor covering spleen cells	83
	5.3.	Serum blocking factors	89
	5.4.	Presence of MTV and anti-MTV-antibodies in the suppressive factor and the blocking serum	89
	5.5.	Discussion	91
Chapter VI	MTV-PROTEIN VACCINE: INDUCTION OF ANTIVIRUS IMMUNITY AND ENHANCEMENT OR INHIBITION OF TUMOUR-SPECIFIC TRANSPLANTATION RESISTANCE	97	
	6.1.	Latency period of tumours after injection and influence of adjuvants	98
	6.2.	Effect of vaccination on the growth of transplanted mammary tumours in BALB/c mice	101
	6.3.	Effect of vaccination on mortality of L1210 leukaemia in DBAf mice	103
	6.4.	MTV-specific immunologic reactivity of vaccinated animals	107
	6.5.	Discussion	109
Chapter VII	MTV-PROTEIN VACCINE: INFLUENCE ON PRIMARY MAMMARY TUMOUR DEVELOPMENT	115	
	7.1.	Influence of an MTV-S protein vaccine on tumour incidence and immunity in female BALB/c, BALB/cfC3H, C3Hf and GR mice	117
	7.2.	Influence of different vaccination schemes on primary tumour development and antibody level in the BALB/cfC3H strain	121
	7.3.	Discussion	123
Chapter VIII	GENERAL DISCUSSION	127	

Summary	135
Samenvatting	141
Acknowledgements	147
Abbreviations	149
References	151
Curriculum vitae	163

THIS THESIS IS BASED ON THE FOLLOWING PUBLICATIONS:

- P. Bentvelzen and P.C. Creemers*: Natural immunity to murine mammary tumor viruses. In: Contemporary topics in immunobiology. Vol. 6, ed. Hanna, Jr., M.G. and Rapp, F., pp. 229-238.
- P. Creemers*: The role of leukocyte subpopulations in the indirect leukocyte adherence inhibition assay in the mammary tumor virus system. *Europ. J. Immunology* 7, 1977, 48-53.
- P. Creemers and P. Bentvelzen*: Cellular immunity to the mammary tumor virus in mice bearing primary mammary tumors. *Europ. J. Cancer* 13, 1977, 503-510.
- P. Creemers and P. Bentvelzen*: The role of T and suppressor cells in MTV-directed cellular immunity. *Europ. J. Cancer* 13, 1977, 261-267.
- P. Creemers, J. Ouwehand, and P. Bentvelzen*: Murine mammary tumor virus protein vaccine: induction of antiviral immunity and inhibition or acceleration of growth of transplanted mammary tumors. *J. Nat. Cancer Inst.* 59, 1977, 895-903.
- P. Creemers and J. Brinkhof*: Factors interfering with cellular immunological responses to the murine mammary tumor virus in tumor-bearing mice. *Int. J. Cancer* 20, 1977, 441-447.
- P. Creemers, J. Ouwehand, and P. Bentvelzen*: Effect of a mouse mammary tumor virus-derived protein vaccine on primary tumor development in mice. In Press. *J. Nat. Cancer Inst.*, June 1978.

CHAPTER I

INTRODUCTION

1.1. Characteristics of the mouse mammary tumour virus

Development of mouse mammary tumours is a complex phenomenon, to which environmental factors, genetic background and the presence of an oncovirus contribute.

The mammary tumour virus (MTV) of the mouse, first discovered by Bittner (1936), is a so-called B-type particle (Bernhard, 1958) and is composed of two major distinguishable elements (Sarkar et al., 1971): a bilayer lipid membrane whose exterior surface is covered with knobbed projections and a viral core. The virus consists of at least 11 different polypeptides; two of the major proteins are glycoproteins with molecular weights of 45,000 to 55,000 and 34,000 to 36,000 Daltons (D); they are usually referred to as gp52 and gp36. The knobs of the viral projections are composed of gp52; the gp36 component is situated within the membrane from which the projections protrude. Three other major polypeptides with molecular weights of approximately 28,000 (p28), 18,000 (p18) and 12,000 (p12) D are associated with the viral core. The main group-specific antigens are gp52 and p28 (Sarkar and Dion, 1975; Sarkar et al., 1976).

The infected mouse mammary tumour cells release mature MTV particles in the intercellular spaces: the RNA-containing core moves into contact with the cell membrane to begin an enveloping process, at the conclusion of which mature MTV-particles bud off from the cell membrane. Intracytoplasmatic type-A particles (iAp) which are also present in mouse mammary tumour cells are considered to be the immature core

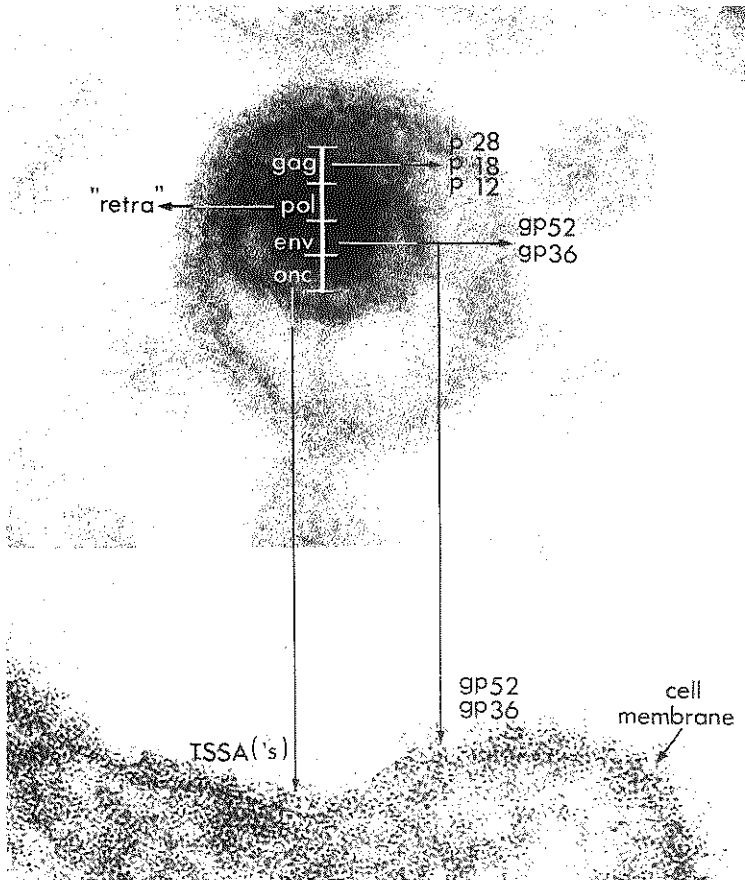
component of MTV (Bernhard, 1960). This is likely since B and A particles share common antigens (Tanaka et al., 1972; Zotter et al., 1976); also, it has been found that after trypsinization of iAp the polypeptides of MTV emerge (Smith and Lee, 1975).

It is generally assumed that, in analogy with the Rous sarcoma virus (Baltimore, 1975), the RNA genome of MTV contains at least four genes. The so-called "gag" gene codes for the core proteins. "Gag" is an abbreviation of group-specific antigens; since not all core proteins are group-specific, this name is, although generally used, not correct. The "pol" gene codes for the RNA dependent DNA polymerase, the so-called reverse transcriptase. The envelope proteins are coded for by the "env"-gene, and the "onc" gene codes for the oncogenic properties of the virus.

The "env"-gene products, so the structural viral envelope proteins, are expressed on the tumour cell membrane. The "onc" gene products are not yet clearly characterized, and are most likely nonstructural virion proteins. In some oncovirus systems, there are indications that the proteins coded for by the "onc" gene are expressed on the tumour cell membrane in the form of tumour specific cell surface antigens [TSSA('s)]. When this model is applied to the MTV-system, the situation will be as is shown in Figure 1.

There are three known modes of natural transmission of MTV in mice:

- a) horizontal transmission: the MTV is introduced into the mouse strain by the maternal milk (Bittner, 1936);
- b) vertical transmission; MTV is present as a provirus in the cellular genomes (Bentvelzen and Daams, 1969).
- c) congenital transmission. Occasionally in utero infection of the zygote by virus in the seminal fluid takes place (Andervont, 1963). Despite several trials, until now no evidence has been found for transplacental transmission of virus (Zeilmaker, 1969).



*Figure 1:
Hypothetical model of MTV-expression*

A B-type particle with the RNA genome, containing four genes: the "gag"-gene, coding for the core proteins p28, p18, and p12; the "pol"-gene, coding for the viral polymerase, "retra"; the "env"-gene, coding for the viral envelope genes gp52 and gp36; and the "onc"-gene, coding for the oncogenic properties of the virus. The products of the "env"-gene and possibly also those of the "onc"-gene [TSSA('s)] are expressed on the tumour cell membrane.

Table 1.

ENDOGENOUS AND EXOGENOUS MTV OF SEVERAL MOUSE STRAINS*

<u>mouse strain</u>	<u>MTV</u>			<u>tumour induction</u>	
	<u>endogenous</u>	<u>exogenous</u>	<u>virulence**</u>	<u>avg. age of mice (weeks)</u>	<u>incidence (+)</u>
C3Hf***	MTV-L	-	+	77	15 %
C3H	MTV-L	MTV-S	++++	31	100 %
BALB/c	MTV-O	-	++++	77	30 %
BALB/cfC3H	MTV-O	MTV-S	++++	36	100 %
GR	MTV-P	-	+++	27	100 %
RIIIIf	?	-	?	82	10 %
RIII	?	MTV-PS	++++	42	90 %
Af	MTV-L	-	+	87	20 %
A	MTV-L	MTV-SV	+++++	18	100 %
DBAf	MTV-L	-	+		

* P. Bentvelzen, 1975; unpublished results 1975-1977.

** See text (page 12)

*** f only stands for foster-nursing on a mouse strain that lacks exogenous virus; when a virus is introduced into a given mouse strain by foster nursing, the strain of the foster mother is also given.

One mouse strain can harbour several substrains of MTV, each of which may be transmitted in a different manner.

1.2. MTV expression and mammary tumour development in different mouse studies

Various mouse strains have been shown to have their own strain-specific MTV variant with its own characteristic expression, virulence, host range and mode of transmission. Table 1 summarizes these properties for several MTV variants.

Except for MTV-P, all virus strains listed in this table induce tumours that show a hormone-independent growth. The exogenous viruses are all transmitted by the the maternal milk. Except for MTV-P, which is transmitted by both the milk and the gametes, all endogenous viruses are heriditarily acquired.

Virulent MTV strains induce 100 % mammary tumours before one year of age when injected into a 4-5-week-old susceptible host; less virulent strains have a longer incubation period. When neonatally or heriditarily acquired, all virulent MTV strains induce a high tumour incidence at a relatively young age in their relevant mouse strains, except for MTV-O, which as far as spontaneous tumours are concerned, only causes a moderate tumour incidence in old retired breeders. MTV-L causes a low tumour incidence in very old mice.

The tumour incidences given here are obtained from animals that were subjected to forced breeding; the resulting hormonal stimulation promotes the development of mammary tumours (Bentvelzen, 1975; unpublished results 1975-1977).

Biochemical evidence for the presence of endogenous viruses has been provided by molecular hybridization studies: when MTV is transmitted vertically, the cellular DNA of all so-

matic cells should contain the complete proviral sequences of this MTV. In the GR mouse strain that carries the to contain identical information (Drohan et al., 1977). DNA of C3H mouse mammary tumours that are induced by the exogenous MTV-S also contain additional proviral MTV-S sequences; however, in the liver these sequences are lacking. The exogenous proviral sequences of MTV-S differ 25 % from the endogenous MTV-L DNA sequences (Michalides et al., 1976).

Nevertheless, infectious MTV-S could be retrieved from the salivary gland, kidney, testis and epididymus of male 6-month-old BALB/cfC3H mice (Bentvelzen and Brinkhof, 1976). This indicates that, besides the mammary gland, also several normal organs are infected by the exogenous virus in the neonatal period. Expression of MTV antigens in normal organs is found in 6-month-old animals of the BALB/cfC3H and GR strains, but not in the BALB/c and C3Hf strains (Haaijman, 1977).

Female mice of the BALB/cfC3H, GR, C3H, A and RIII strains all produce high levels of MTV in the milk at a young age; these strains are therefore considered as high MTV expressors. Mouse strains that produce only moderate amounts in their milk at a late age (Af, C3Hf, DBAf, BALB/c and RIIIif) are referred to as low MTV expressors.

When no tumour is present, blood plasma levels of gp52 are low in males and females of both high- and low-MTV-expressing strains. The gp52 levels are not affected by lactation. Only shortly before the tumour becomes clinically detectable does the level increase (Ritzi et al., 1976).

On mammary tumour cells, MTV-antigens are not restricted to budding B-particles but are also present on other sites of the plasma membranes (Calafat et al., 1974). Recently, specific antisera against the MTV polypeptides gp52, p28 and p12 became available in our laboratory and three cell lines were tested for the presence of these antigens on the cell membrane: the virus-producing C3H derived cell line called

C3HMT/cl.11, the MTV-P producing GR mouse ascitis leukaemia, called GRSL and the DBAf MTV-containing L1210 ascites leukaemia. All cell lines expressed gp52 and to a lesser extent p12 on the membranes, but no clear-cut evidence for the expression of p28 on mammary tumour cell membranes could be found (Westenbrink et al., 1978).

On incubation with anti-MTV-antiserum, mammary tumour cells show antigenic redistribution and subsequent shedding of the MTV antigens from the cell surface; normal tissue antigens on these tumour cells did not show this phenomenon (Calafat et al., 1976). This finding will have serious consequences for the effectiveness of the immune reaction of the animals, since tumour antigens in a soluble form will compete with the tumour for the immune effector processes (Alexander, 1974).

1.3. Immunity to MTV in healthy animals

Although it has been a widely held opinion for a long time that healthy mice are immunologically tolerant to endogenous and exogenous oncoviruses, this concept has been challenged by several recent reports which demonstrate the occurrence of natural immune responses to these viruses.

In the MTV system, spontaneously occurring antibodies reacting specifically to MTV have been demonstrated in tumour-free animals in strains with a high mammary tumour incidence. Some authors also describe the appearance of antibodies in female tumour-free mice in strains with a low tumour incidence (Muller et al., 1971). The concentration of antibodies increases with age (Ihle et al., 1976). In addition, the occurrence of natural antibodies against iAp has been reported (Zotter and Muller, 1977).

MTV-specific cellular immunity has also been observed in mice long before the onset of tumour growth. Very extensive investigations have been performed by Blair (1976) with the

microcytotoxicity assay in BALB/cfC3H and BALB/c mice. According to her findings, spleen cells of BALB/cfC3H mice become reactive within one month after birth. This response is mediated by T cells. She also observes antibody-dependent cell-mediated cytotoxicity (ADCC) mediated by null cells and factors which block the T cell mediated target cell lysis. BALB/c mice become reactive only after four months; she postulates that this response is initiated by horizontal transmission of the exogenous MTV-S.

It must be taken into account, however, that the microcytotoxicity assay performed in microtiter plates is technically cumbersome and not very reliable (Howell et al., 1974; Brown et al., 1976). Moreover, Blair's results could not be repeated in several laboratories, including our own (unpublished results). Therefore the relevance of these findings remains unclear.

In contrast to Blair, Lopez et al. (1976) and Sigel et al. (1976), who measured reactivity with lymphocyte stimulation and migration inhibition assays, report that BALB/c mice are reactive, whereas BALB/cfC3H mice fail to respond; for the latter, they suggest a form of unresponsiveness, since lack of reactivity can be changed to reactivity by a tumour implant. This is in accord with the findings of Gillette and Lowrey (1976) with a cytostasis assay. They found that reactivity was greater in lymphoid cells from low MTV expressors than in syngeneic high MTV expressor strains. Newborns were all negative. Although cytostasis was not mediated by theta-positive cells, cells from nude athymic donors did not respond; they conclude therefore that T cells are required for the initiation of this form of anti-MTV-immunity.

Although all authors agree that immune responses in tumour-free animals are much lower than in tumour bearing mice, it can be concluded from these findings that mammary tumours progress in the face of a significant response to MTV-induced antigens.

1.4. Immunity to MTV in tumour-bearing animals

There are many reports on the presence of precipitating antibodies to MTV in mice bearing palpable tumours. Results with radioimmunoassay (RIA) (Ihle et al., 1976) revealed much higher titers in tumour-bearing C3H females than in normal animals. Muller et al. (1971) also found that, except for the GR strain, more tumour bearing than normal animals had anti-MTV antibodies in their serum. Immune complex deposits have been found in the glomeruli of tumour bearing RIII mice (Pascal et al., 1975).

Stolfi et al. (1975) detected that sera from tumour bearers express complement-dependent cytotoxic activity, but only when high dilutions were used. They postulate that at low dilutions the cytotoxic reaction is prevented by an inhibitor, but have not investigated this further.

With respect to cellular immunity to MTV in tumour bearers, Blair (1976), using the microcytotoxicity assay, gives an extremely complex picture: In BALB/cfC3H mice, the ADCC becomes undetectable during tumour growth and is not found in females bearing large progressively growing tumours. The factors that block T cell activity remain in the circulation: in addition, 2 other serum factors become detectable: a) a factor that blocks ADCC; and b) an antibody not directed to MTV but tumour-specific, which recruits normal cells to cytotoxicity. Also for these results, however, the considerations concerning the reproducibility of this test must be taken into account (page 16). In two almost completely identical articles (Roubinian et al., 1976; Roubinian and Blair, 1977), this group also reports that tumour growth is inhibited by incomplete thymectomy, whereas complete removal of the thymus leads to promotion of tumour growth. Besides T cell dependency of the anti-MTV-reaction, this also points to the presence of T suppressor cells.

Sigel et al. (1976) and Lopez et al. (1976) found higher MTV-specific migration inhibition and leukocyte stimulation reactions in tumour bearers than in normal animals. They do not mention a change in reactivity during tumour growth. This group also reports on the emergence of a complement-receptor-positive T cell population during development of the tumours (Epstein et al., 1976).

In the investigation reported in this thesis, the observation concerning the T cell dependency of anti-MTV and anti-tumour cell cellular immune reactivity was confirmed.

During the study of cell-mediated immune reactions in tumour-bearing mice, it became evident that this reactivity declines gradually during tumour growth and becomes severely impaired. In Gross leukaemia virus and Moloney sarcoma virus induced tumour systems, this immune deficiency was caused by cells with adherent properties, which suppressed cell proliferation induced by stimulation with plant lectins (Kirchner et al., 1974; 1975; Glaser et al., 1975). Kruisbeek and Van Hees (1977) observed this phenomenon in rats bearing transplanted bladder carcinomas. In the MTV system, we found such suppressor cells inhibiting MTV-specific cell proliferation. However, in spleen cell suspensions of animals bearing large tumours removal of these suppressor cells was not sufficient to restore the specific anti-MTV reactivity. Also, fluctuations in the inhibition by the suppressor cells during tumour growth did not show a reverse correlation with the variation in the responsiveness of lymphoid cells. The decline in cellular reactivity to MTV during tumour growth must therefore also be due to other factors.

In various systems, it has been shown that nonresponsiveness of lymphoid cells during tumour growth can be eliminated by either slight trypsinization or extensive washing of the cells (Hattler and Soehnlén, 1974; Kjaer, 1976; Grosser and Thompson, 1976). In this study, these procedures have been successfully applied to restore

reactivity to MTV. In addition, the presence of serum factors that block cellular reactivity has been established. The possible nature of these inhibiting factors was investigated.

1.5. Vaccination experiments

Several populations of chickens show a very high incidence of lymphoma's (Marek's disease); they can be completely protected by vaccination with the attenuated causative herpes virus. It is highly interesting to know whether this approach is also useful for prophylaxis of tumours caused by RNA oncoviruses.

Until recently, most vaccination studies have been performed with inactivated malignant cells or whole virus. However, there is widespread concern about the dangers of introducing materials that may contribute to the development of neoplasms. For this reason vaccination studies on oncoviruses focus more and more on purified viral subunit proteins.

Successful vaccination is still difficult to achieve. Despite a large number of trials with malignant cells, oncoviruses and viral subunits, this has resulted in only a few successes in terms of tumour prophylaxis. In view of the existence of natural immunity against many oncoviruses, this is not surprising: immunization in these cases represents an alteration of an existing immune status rather than induction of immunity.

Promising results have been described for vaccination against feline leukaemia virus (FeLV) in cats: protection against challenge with live virus has been achieved with feline oncovirus associated cell membrane antigen (FOCMA) and with ultraviolet (UV) light inactivated feline sarcoma virus (FeSV) (Jarrett et al., 1975). Hunsmann et al. (1975) report that vaccination of a low-leukaemic mouse strain

with the purified major surface glycoprotein gp71 of Friend leukaemia virus (FLV) resulted in significant lower spleen weights after a later virus challenge in treated animals as compared to control mice.

It was later on reported, however, that mice immunized with FLV are resistant to a subsequent virus challenge only when they have become infected with the FLV-associated lymphatic leukaemia virus (Bendinelli, 1977). The authors reporting on FeLV and FLV all claim that protection was associated with high antibody titers.

In other oncoviral systems, however, attempts at vaccination led to a more pessimistic attitude. Gross leukaemia virus-induced tumours in rats showed a significantly enhanced growth following sensitization to soluble tumour antigen; this was associated with a depression of cell-mediated immunity (Rao and Bonavida, 1976). A KCl extract derived from a murine tumour cell line containing both Moloney leukaemia virus and Moloney sarcoma virus had both immunogenic and immunosuppressive capacities (Brandschaft et al., 1976).

In the MTV system, several attempts have been made to vaccinate mice with mammary tumour cells as well as with purified virus preparations. Injection of mammary tumour cells which were inactivated by X-radiation into C3Hf mice that had been surgically cured of a mammary tumour resulted in specific protection against later tumour challenge (Vaage and Agarwal, 1976). Injection of an extract from a C3Hf-derived tumour cell line containing TSSA led to a reduction in spontaneous tumour development in C3Hf mice (Irie and Irie, 1971).

The first indications that vaccination may be complicated by suppressive immune mechanisms emerged when it was found that immunization with mammary tumour tissue or virus and complete Freund's adjuvant (FA) lead to the accelerated appearance of spontaneous tumours in neonatally infected mice (Hinsch and Iversen, 1961; Bentvelzen et al., 1970).

Tumour growth enhancing phenomena were also observed when the influence of a derivative of BCG, called MER, was tested: treatment with MER alone prior to tumour transplantation caused acceleration of tumour growth in BALB/c and BALB/c mice. MER treatment combined with specific tumour immunization did not result in enhancement of tumour development. However this combined treatment had no added beneficial effect over immunization with tumour alone (Jacobs and Kripke, 1974).

When purified MTV preparations are used, protection against tumour development is almost uniformly reported: Formalinized MTV-S with or without FA gives protection in BALB/c mice (Burton et al., 1969). Inoculations with live, formalinized or ether-treated MTV-S preparations in GR mice at the age of 4 - 7 weeks all delayed tumour development; vaccination at a later age gave less protection. Live MTV-P had no effect, in contrast to H₂O₂-treated MTV-P preparations (Van der Gugten and Bentvelzen, 1969). Formalinized RIII virus resulted in protection against spontaneous tumours in the low-MTV-expressor RIII^f and Af mouse strains. A dose of 1 µg in combination with FA protected C57BL mice against later RIII virus challenge (Charney et al., 1976).

Stutman (1976) reports a moderate protective effect in both C3H and C3H^f mice after vaccination with formalinized MTV; however, when soluble antigens extracted from C3H mammary tumours were used, there was an acceleration of tumour appearance.

We are presently engaged in a study concerning tumour prophylaxis by use of MTV subunit protein vaccines and have obtained protection using low doses of an MTV protein fraction that was enriched for gp52.

For the development of a suitable vaccination procedure, we first investigated the induction of transplantation resistance to MTV-containing neoplasms. We used BALB/c mice which were challenged with MTV-O-containing mammary

tumours and DBA_f mice which were challenged with the MTV-containing L1210 ascitic leukaemia (Radzikowski et al., 1972).

As an adjuvant, we used alum, which is known to enhance antiviral immunity (Mautner and Willcox, 1974), and an interphase material (IPM) isolated from *Mycobacterium smegmatis* which has a considerable antitumour activity in itself (Lamensans et al., 1975).

Cellular and humoral immune responses to MTV were monitored in order to obtain insight into the immune mechanisms evoked by the different vaccination procedures.

In the view of the protection against transplanted tumour cells that occurred after vaccination with a low dose of the MTV-protein fraction, we studied the influence of a low fixed dose (10 µg) of this vaccine on primary tumour development in the BALB/c, C3H, BALB/cfC3H and GR strains. In addition, in BALB/cfC3H mice we studied the influence of the administration of booster doses on primary tumour development.

1.6. Rationale for the investigation: relation of the mouse model to human breast cancer

The lack of rejection of breast tumours in both man and mice is most probably due to an insufficient response of the immune system. A main reason for studying the immune response to mammary tumour antigens in mice is to obtain some insight into problems concerning immune therapy of humans suffering from breast cancer, or vaccination of high risk groups. Therefore, antigenic and epidemiological relatedness between mouse MTV and human breast cancer antigens is of utmost importance.

The first evidence for possible involvement of an oncovirus in human breast cancer was found in 1974 when McGrath et al. and Lasfarques and Moore detected virus-like particles, as well as reverse transcriptase in human breast cancer cell lines. The particles showed cross-reactivity to

MTV (McGrath et al., 1974). Zachrau et al. (1976) presented evidence that approximately 65 % of human breast cancer tissues contained a prominent protein fraction with a molecular weight of 47,000 to 55,000 D, whereas such component could not be found in benign breast tissue. An indication for an epidemiological similarity between MTV in mice and the human breast cancer particle is provided by the finding of particles in milk from apparently healthy humans (Moore et al., 1970). The major core protein of these particles has a molecular weight of 27,000 D and is electrophoretically identical to the major core protein of MTV (Furmanski et al., 1976).

There is some evidence that in human breast cancer also particles related to Mason-Pfizer monkey virus (MPMV) occur: the RNA of human breast cancer particles possess detectable homology to the RNA of MTV (Axel et al., 1972) and of MPMV (Colcher et al., 1974). Also the purified reverse transcriptase of the human breast cancer particle cross-reacts immunologically with the MPMV enzyme (Ohno and Spiegelman, 1977).

Other evidence for antigenic relationships between MTV proteins and those of the human breast cancer particle is provided by immunological studies: Muller et al. (1976) detected antibodies in the sera of breast cancer patients which were specifically reactive to iAp in mouse mammary tumour cells.

Fifty per cent of breast cancer patients and 25 % of normal woman harbour lymphoid cells that react to RIII mouse milk (Cunningham-Rundles et al., 1976); this reactivity was associated with preferential cross-reactivity to C3H-MTV and human autologous and homologous breast cancer tissue that contained gp55 resembling mouse MTV gp52. Minimal cross reactivity was found with gp50 from strain A MTV, RLV and with human breast cancer tissue that had no mouse MTV-gp52-like protein (Black et al., 1976). By means of membrane immunofluorescence with anti-MTV gp52 anti-

serum, gp52 could be demonstrated on peripheral lymphocytes of 14 out of 20 breast cancer patients, while only 2 of 10 normal age-matched women had reactive lymphocytes (Wiseman et al., 1977).

Another characteristic shared by the cell surface antigens of mouse and human mammary tumour cells is the phenomenon of antigenic modulation: upon incubation with an antiserum specifically reactive to human breast cancer antigens, breast cancer cells show redistribution of antigens and subsequent shedding of these antigen-antibody complexes (Nordquist et al., 1977).

For a rational approach to specific immune manipulation, it is necessary to know the cause of the derailment of the immune reaction during tumour growth. Therefore, we first studied the immune response to MTV in tumour-bearing mice. During these studies, we found immunological indicators that were relevant for tumour survival in untreated mice. Besides measuring the resistance to primary and transplanted tumours, these indicators were used to evaluate the effectiveness of several vaccination schemes.

CHAPTER II

TECHNICAL APPROACH

2.1. *Animals*

The following inbred mouse strains were used for the studies reported here:

GRS 11/A Rij f, further referred to as GR. Genetics: c, H-2^d_x. Origin: Gr nder, in 1955 to Amsterdam, in 1969 to Rijswijk. Inbreeding generation at the Radiobiological Institute (RBI), Rijswijk: F21-F24.

C3Hf/Lw Rij f, further referred to as C3Hf. Genetics: +, H-2^k. Origin: 1920 Strong, in 1930 to Andervont, in 1941 to Heston, to Law, in 1965 to Rijswijk. Inbreeding generation at RBI: F38-F41.

BALB/c AN Crl Rij f, further referred to as BALB/c. Genetics: b, c, H-2^d. Origin: 1913 Bagg, in 1923 to McDowell, in 1932 to Snell, to Andervont, to ?, to Battelle Memorial Institute, in 1966 to Charles River Laboratories, in 1968 to Rijswijk. Inbreeding generation at the RBI: F25-F28.

BALB/c foster nursed on C3H, further referred to as BALB/cfC3H. This mouse strain has been established in our laboratory in 1970 by Dr. P. Bentvelzen.

DBA/2 f, further referred to as DBAf. Genetics: unknown. Origin: from Law, in 1962 to the Medical Biological Laboratory, Rijswijk, from which we received these mice.

C57BL/Ka Lw Rij f, further referred to as C57BL. Genetics: a, H-2^b. Origin: 1921 Little, to Strong, in 1947 to Kaplan, to Law, in 1965 to Rijswijk. Inbreeding generation at RBI: F30-F33.

All strains were conventional from a gnoto-biological stand point.

In the GR, C3Hf, BALB/c and BALB/cfC3H strain, mammary tumours were induced by forced breeding. The mice received water and food (AM₂, Hope Farms, Woerden, The Netherlands) *ad libitum*.

Immunized mice were obtained by intraperitoneal (ip) injection with 1 µg MTV precipitated on alum (preparation: see 2.9). Male mice not older than 10 weeks served as normal control animals. Serum was collected from the retro-orbital plexus under ether anaesthesia; for the experiments on cellular immunity, the animals were killed by cervical dislocation.

For the study of the influence of histoincompatibility in the leukocyte adherence inhibition (LAI) assay, 7-year-old male chimpanzees that had been in our colony (Primate Center TNO, Rijswijk) for four years were used.

2.2. Virus purification

The standard strain of the MTV (Teramoto et al., 1974) was isolated from primary BALB/cfC3H mammary tumours in the following way: Fifty g of tumour was homogenized in 500 ml phosphate buffered saline (PBS) (pH 7.2) in a Sorvall omnimixer (Ivan Sorvall Inc., Norwalk, Conn.) for 50 sec at 14,000 rpm. The homogenate was centrifuged for 15 min at 20,000 x g (Sorvall RC2-B, GSA rotor, 12,500 rpm). This centrifugation was repeated with the resuspended pellet. To the combined supernatants EDTA was added to a final concentration of 1 mM. After centrifugation for 45 min at 100,000 x g (Beckman 35 N rotor, 35,000 rpm; Beckman Instrument, Inc., Fullerton, Calif.), the pellet was resuspended in 1.5 mM Tris HCl (pH 7.2) and 0.025 M sucrose. A total volume of 30 ml virus suspension was centrifuged for 20 min at 20,000 x g (Sorvall RC2-B, SS34 rotor, 12,500 rpm). This centrifugation step was repeated with the resuspended pellet.

Hereafter 15 ml of the combined supernatants was

layered on a discontinuous sucrose gradient composed of 4 ml 50 % sucrose, 12 ml 35 % sucrose, and 6 ml 20 % sucrose (wt/wt) in 1.5 mM Tris-HCl (pH 7.2). After centrifugation for 180 min at 100,000 x g (Beckman SW27.1 rotor, 27,000 rpm), a sharp light-scattering band on the 50 % layer was separated, diluted, and layered on a preformed linear sucrose gradient (20-50 %) and centrifuged for 180 min at 100,000 x g. The sharp visible band at a density of 1.16-1.18 g/cm³ containing the virus was collected, diluted, and centrifuged for 45 min at 45,000 xg (Beckman 50 Ti-rotor, 40,000 rpm). The virus pellet was resuspended in 1.5 mM Tris-HCl (pH 7.2) in 0.025 M sucrose and stored at -70° C until use. All isolation steps were performed at 4° C. The yield was approximately 1 mg virus protein per 10 ml of tumour. The polyacrylamide gel electrophoresis (PAGE) analysis of virus isolated in this way showed the same protein pattern as virus derived from cells grown in tissue culture (Westenbrink et al., 1977). RLV was isolated from leukaemic spleens of BALB/c mice in the same manner. Virus content was estimated on the basis of the amount of protein, as measured by the Lowry method.

2.3. *The direct leukocyte adherence inhibition (LAI) assay*

The direct LAI assay for demonstrating immunologic cellular reactivity to certain antigens was developed by Halliday and Miller (1972). They observed that the proportion of cells adhering to glass surfaces was reduced if antigen to which the animals were sensitized was added. In this test system, the blocking and enhancing properties of serum could also be established. Later, it was reported that the LAI assay may be regarded as a dependable *in vitro* test for determining immunologic reactivity to tumour specific antigens in the case of melanoma (Halliday et al., 1975) and breast cancer (Grosser and Thompson, 1975). For these reasons, we decided to use the direct LAI assay to monitor

MTV-directed cellular immunity in mice bearing mammary tumours.

The assay was performed in the following manner: Immediately after killing the mice were injected intraperitoneally (ip) by means of a Pasteur pipette with 5 ml of ice-cold medium (RPMI 1640 with 100 IU penicillin and 100 µg streptomycin/ml and 5×10^{-5} M 2-mercaptoethanol) to which heparin (5 IU/ml) was added. The fluid was withdrawn and re-injected several times and then collected into glass tubes. The cells were centrifuged once at 200 x g for 10 min. Washing was restricted since we noticed that peritoneal cells become unresponsive in the direct LAI-assay after repeated washing. Cells of immunized DBAf mice showed a significant reduction of adherence of about 30 % upon incubation with MTV after one washing. The activity decreased to zero after the cells had been washed four times.

The cells were then resuspended in medium containing 7% foetal calf serum (Flow, Irvin, Scotland). The final cell concentration was adjusted to approximately 40×10^3 /ml. Then antigen was added and 20 µl of the suspension were pipetted into the wells of Falcon microtest 3034 plates.

The wells for the control and test cells were alternating. This was done because it was sometimes observed that more cells adhered in the wells at the edges of the plates than in the middle. The amount of cells that adhered in the plates was also very low. The plating efficiency could be increased by incubation of the plates in PBS for two days. Greiner microtest plates (Nürtingen, Germany) showed these disadvantages to a lesser extent. Different batches of plates may be the cause that the plating efficiency in different experiments sometimes varies considerably.

When the wells were filled, the plates were incubated in a humidified atmosphere with 5 % CO₂ for 2 h at 37° C. Thereafter, the plates were washed three times in PBS and the adherent cells were fixed, stained and counted.

At least eight wells for each antigen were counted. To avoid non-specific reactions of the indicator cells, purified RLV was used as a specificity control throughout. Reactivity was expressed as per cent reduction of adherence, which was calculated according to the formula $(1 - \frac{a}{b}) \times 100 \%$ in which a is the average number of cells adhering in the presence of MTV, and b those adhering in the presence of RLV. Statistical significance was established by means of the two-tailed Students' t-test.

For our MTV-S antigen preparation, isolated from BALB/cfC3H mammary tumours, the optimal antigen concentration was 0.1 μg MTV/ml. This is illustrated on fig. 2. For a batch RIII virus, isolated from milk (obtained through the Office of Resources and Logistics, Virus Cancer Program, National Cancer Institute), it was however observed that the optimal antigen concentration was 0.01 μg MTV/ml. This may be due to the fact that the antigen concentration is based on the

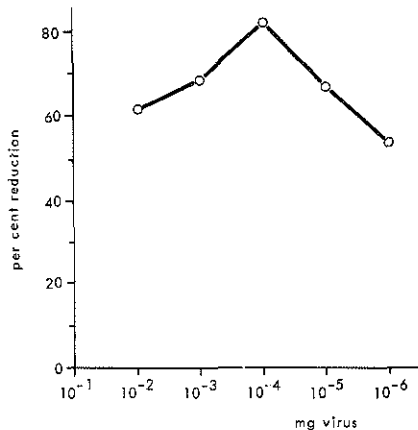


Figure 2:

Optimal concentration of MTV in the direct LAI-assay

MTV-specific leukocyte adherence inhibition of peritoneal cells derived from immunized C57BL mice in the presence of varying concentrations of MTV, as compared to the RLV control value. This figure represents the results of one of three experiments. The results from all three experiments were similar.

Table 2

DIRECT LEUKOCYTE ADHERENCE INHIBITION IN NORMAL MALE MICE

Mouse strain	Incubation with medium	Incubation with RLV	Incubation with MTV	Significance of difference be- tween RLV and medium control	Significance of difference be- tween RLV and MTV
	Average no. of adherent cells \pm SE*				
BALB/c	29.2 \pm 1.6	28.6 \pm 3.2	30.0 \pm 3.5	NS**	NS
BALB/cfc3H	60.8 \pm 4.1	43.7 \pm 3.5	42.3 \pm 2.3	p < 0.001	NS
GR	25.6 \pm 4.2	19.6 \pm 2.8	20.3 \pm 1.8	NS	NS

* SE: standard error

** NS: not significant

amount of protein. Also different amounts of group-specific antigens in varying MTV-variants may play a role. Therefore, for every batch of MTV the optimal antigen concentration was again established.

When pooled cells derived from 3 normal animals were tested, a significant difference was sometimes found between the number of adherent cells in cultures incubated with 0.1 μ g RLV and those to which no antigen was added. However, no appreciable differences were found between cultures incubated with RLV and MTV. For the strains BALB/c, BALB/cfC3H and GR, this is illustrated in Table 2. Normal C57BL and DBA mice and BALB/c mice sensitized to a plasmacytoma also do not respond to MTV. Neither was a significant difference ever found when normal cells were incubated with 0.1 μ g RLV, as compared to the same amount of ovalbumin. C57BL and DBA mice immunized with RLV were positive to RLV and negative to MTV.

2.4. The indirect LAI-assay

Holt et al. (1975) reported that the LAI reaction can be abolished by pretreatment of the cells with anti-theta serum and complement. He also observed that the inhibition of adherence can be transferred to cells from nonimmunized animals by the supernatant of spleen cells incubated with the antigen. Therefore, he concluded that a soluble factor, the T cell-dependent leukocyte adherence inhibition factor (LAIF) is responsible for the inhibition of adherence. We developed the indirect LAI assay, in which LAIF, produced by spleen cells upon incubation with MTV is transferred to normal indicator cells.

This test was performed under sterile conditions. Spleen cells were taken aseptically and teased apart by forceps in a petri dish containing medium. The debris was then allowed to settle in a tube. The supernatant containing the cells was centrifuged twice at 26 x g for 10 min to

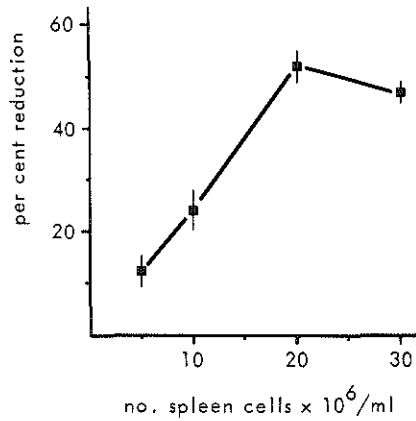


Figure 3:
Optimal spleen cell concentration per ml in the indirect LAI-assay

MTV-specific LAIF production at varying spleen cell concentrations. Pooled spleen cells from five immunized DBA/1 mice were used. Incubation time of the cultures was 24 h. Vertical bars indicate SE of the per cent reduction of adherence. Shown in the figure are the results of one out of three experiments. The results from the three experiments were similar.

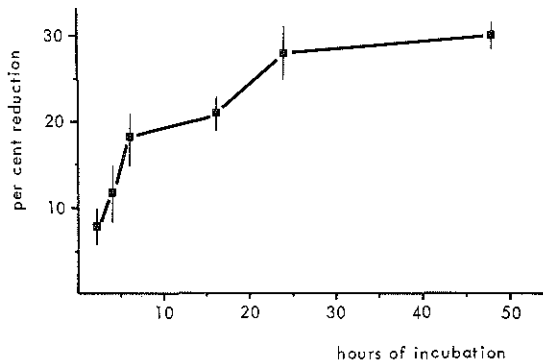


Figure 4:
Optimal incubation time of cultures in the indirect LAI-assay

MTV-specific LAIF production by pooled spleen cells derived from five immunized DBA/1 mice in the presence of 1 μ g MTV, after varying incubation times. Vertical bars indicate SE of the per cent reduction of adherence. This experiment has been repeated with similar results.

remove erythrocytes and afterwards centrifuged twice at 200 x g for 10 min. This was done to remove LAIF that could have been produced by the cells *in vivo* through stimulation by the expression of MTV-antigens in the animal.

After counting, the spleen cells were resuspended in medium supplemented with 10 % foetal calf serum. The cultures (1 ml) were incubated in plastic tubes (Falcon, 12 x 75 mm) for 24 h with 0.1 µg antigen in a humidified atmosphere with 5 % CO₂. The cells were subsequently spun down and the supernatant was removed.

As indicator cells fresh peritoneal cells derived from normal animals were used. The cells were washed 4 times at 200 x g for 10 min.

The indicator cells were then suspended in the supernatant of the spleen cells to a final concentration of approximately 40 x 10³ cells/ml, and 20 µl was again pipetted into the wells of microtest plates. The plates were then treated as described for the direct LAI test; percentage reduction of adherence was also calculated in the same way. In this assay the optimal antigen concentration for MTV-S derived from BALB/cfC3H mammary tumours was again 0.1 µg.

Optimal conditions for the indirect LAI assay. In the indirect test, the highest response was obtained if the spleen cell concentration was 20 x 10⁶/ml (Figure 3). On prolonging the incubation time of the spleen cells, LAIF production also increases. A maximum is reached after 24 h (Figure 4).

We compared the reactivity measured with the direct and the indirect method of pooled cells from immunized DBAf mice. The indirect assay was performed using the optimal spleen cell concentration (20 x 10⁶/ml) and incubation time (24 h). The results are shown in table 3. Reactivity is higher when the indirect method is used. From the results it is clear that the SE values of both assays are

Table 3

COMPARISON OF REDUCTION OF ADHERENCE OF CELLS FROM THE SAME IMMUNIZED ANIMALS AS MEASURED BY THE DIRECT AND THE INDIRECT LAI ASSAY

Exp. no.	Direct LAI-assay			Indirect LAI-assay		
	Incubation with RLV	Incubation with MTV	Reduction of adherence (%)	Incubation with RLV	Incubation with MTV	Reduction of adherence (%)
	Average no. of adherent cells \pm SE**			Average no. of adherent cells \pm SE		
1	23.6 \pm 2.8	21.0 \pm 1.3	11	65.3 \pm 0.9	40.5 \pm 2.3	38*
2	25.4 \pm 1.2	22.0 \pm 0.4	13	64.5 \pm 3.2	43.2 \pm 2.5	33*
3	54.3 \pm 5.7	38.6 \pm 0.8	29*	16.3 \pm 1.5	8.5 \pm 2.1	48*
4	28.2 \pm 0.7	21.9 \pm 1.9	23*	75.8 \pm 3.8	34.1 \pm 0.6	55*
5	78.3 \pm 3.4	65.8 \pm 2.1	16*	91.8 \pm 5.2	58.6 \pm 2.8	37*

* : statistically significant ($p < 0.010$)

** SE: standard error

similar. Also, on several occasions we tested the reproducibility of both assays; in both tests the results did not deviate from each other by more than 5 % (results not shown). For these reasons, it is concluded that the indirect assay is more sensitive than the direct test.

When the direct and indirect LAI tests, both performed with peritoneal cells, are compared at an incubation period of 2 h, a similar reactivity is found in both tests. In the indirect LAI assay spleen cells gave better results than peritoneal cells. This is illustrated in table 4. LAIF-production by spleen cells may be higher because the proportion of T cells is higher in spleen cells than in peritoneal cells. It could be anticipated that also in the direct assay spleen cells will probably give better results. However, this could not be tested: When spleen cells were used as indicator cells it was noticed that standard deviations became larger, probably due to overcrowding of the wells by non-adhering cells.

The greater sensitivity of the indirect assay as compared to the direct test is also due to the longer incubation period used. It is impossible to use such long incubation times in the direct assay, since the standard deviations of the results increase greatly with prolonging the incubation time. In addition, the role of spleen cell subpopulations and lymphokines can be investigated more conveniently by means of the indirect LAI-assay.

For these reasons, the indirect assay was preferred over the direct assay in later studies (Chapters IV and V).

Influence of histoincompatibility. An interesting question was whether LAIF can be transferred to the adherent cells from mice of other strains or cells from a distantly related species. For this reason, it was investigated whether LAIF derived from spleen cells of immunized DBA/f mice could be transferred to the adherent cells of BALB/c

Table 4

MTV-SPECIFIC LAIF PRODUCTION BY IMMUNE CELLS AS MEASURED BY THE DIRECT AND THE
INDIRECT LAI ASSAY DURING A 2 H INCUBATION PERIOD

Method and cell population	Experiment 1		Exp. 1	Exp. 2	Exp. 3
	Incubation with RLV Average no. of cells \pm SE*	Incubation with MTV adherent cells \pm SE*			
Direct LAI					
Peritoneal cells	14.8 \pm 0.5	10.8 \pm 0.9	27	20	15
Indirect LAI					
Peritoneal cells	29.3 \pm 1.3	21.0 \pm 0.9	28	18	19
Spleen cells	15.8 \pm 0.6	10.0 \pm 1.0	37	31	27

* SE: standard error

** : all values for per cent reduction of adherence are significant ($p < 0.010$)

and C57BL mice and to those of chimpanzees. For the testing of the chimpanzees, lymphoid cells from pooled peripheral blood of three animals were purified by Ficoll-Hypaque sedimentation and washed 4 times in medium. They were then suspended in the supernatant to a final concentration of 10^6 cells/ml; 20 μ l were used/well. The MTV-specific LAIF production by the DBAf-derived immune spleen cells as measured when lymphoid cells from other mouse strains and the chimpanzees are used as indicator cells is shown on Table 5.

From these results, it is clear that histoincompatibility within the same species does not interfere in this test. Thus, when only small numbers of a certain mouse strain to be tested were available, indicator cells were derived from another mouse strain. The murine LAIF cannot be transferred to another species.

Testing for blocking or enhancing capacities of serum.

To determine the influence of serum on the indirect LAI reactivity, 0.1 ml of serum to be tested was added to 0.9 ml cultures of immune spleen cells and 0.1 μ g of MTV; 0.1 ml of syngeneic mouse serum served as a control. Blocking or enhancing capacity of serum was expressed by the formula: quotient $y = TS/NS$, in which TS is the number of adherent cells in the wells containing test serum and NS that in the wells containing normal serum. Since no specificity control was included, the blocking or enhancing properties of the sera that is measured may be partly aspecific.

2.5. Leukocyte stimulation (LS) test

This test, known as mitogen stimulation is in common use for measuring non-specific immune competence with plant lectins. Incorporation of ^{14}C -thymidine is then taken as

Table 5

MTV-SPECIFIC LAIF PRODUCTION BY DBAf-DERIVED IMMUNE SPLEEN CELLS MEASURED WITH PERITONEAL CELLS FROM OTHER MOUSE STRAINS, AND WITH LYMPHOID CELLS OF CHIMPANZEES AS INDICATOR CELLS

	Origin of indicator cells	Exp. 1		Exp. 1	Exp. 2	Exp. 3
		Incubation with RLV Average no. of adherent cells \pm SE*	Incubation with MTV Average no. of adherent cells \pm SE*	Reduction of adherence (%)**		
A	DBAf mice	79.5 \pm 2.3	63.5 \pm 1.5	20	24	17
	BALB/c mice	30.4 \pm 1.1	25.1 \pm 1.2	17	21	20
	C57BL mice	50.2 \pm 2.6	40.1 \pm 1.3	20	19	15
B	DBAf mice	34.3 \pm 1.6	11.8 \pm 2.0	66	28	43
	chimpanzees	96.0 \pm 2.7	96.7 \pm 4.2	0	4	3

* SE: standard error

** : all values, except those of cells from chimpanzees, are significant ($p < 0.010$).

a measure for cell proliferation. The LS test can also be used to measure a specific immune response. Spleen cell preparations were obtained as described above. When lymph node cells were tested, they were teased apart by forceps in a petri dish containing medium and spun 4 times at 200 g for 10 min. After counting, the cells were resuspended (2.5×10^6 /ml) in medium supplemented with 20 % FCS. The cultures (1 ml) were incubated for 80 h in plastic tubes (75 x 12 mm, Falcon) in the presence of antigen at 37° C in a humidified atmosphere with 5 % CO₂. After this, 0.15 μ Ci of 2-¹⁴C-thymidine was added to each culture. Twenty-four h later, the cells were collected by suction onto glass fibre filters (Whatman GF/A, Maidstone, England) and washed two times; incorporation of ¹⁴C-thymidine was measured in a liquid scintillation counter.

The cultures were run in triplicate. Percentage of stimulation was calculated according to the formula $a - b/b \times 100 \%$, in which a = average number of counts/min (cpm) in cultures incubated with MTV and b that in cultures incubated with RLV. Statistical significance was established by means of the two-tailed Students't-test.

With lymphoid cells from immunized mice, the optimal conditions for stimulation, as measured by ¹⁴C-thymidine uptake, are 80 h of incubation (Figure 5) and 0.1 μ g viral protein when MTV-S, isolated from BALB/cfC3H mammary tumours was used (Figure 6). Also in this test it was necessary to establish the optimal concentration of antigen for each new batch of antigen.

From repeated experiments (3 for every mouse strain), it became clear that spleen cells from normal animals sometimes show an enhanced response with RLV as compared to the medium control experiment. This is demonstrated in Table 6. In this case also, the same values are found for RLV and MTV. This also holds true for RLV as compared to ovalbumin. Therefore, in this test RLV is again taken as the specificity control.

Table 6

¹⁴C-THYMIDINE INCORPORATION OF SPLEEN- AND LYMPH-NODE CELLS OF NORMAL MALE MICE

Mouse strain	Incubation with medium cpm \pm SE	Incubation with RLV cpm \pm SE	Incubation with MTV cpm \pm SE	Significance of difference between RLV and medium control	Significance of difference between RLV and MTV
BALB/c	2.010 \pm 298	1.992 \pm 146	1.708 \pm 312	NS**	NS
GR	2.078 \pm 266	3.862 \pm 308	3.366 \pm 352	p < 0.025	NS
BALB/cfC3H	3.619 \pm 72	7.912 \pm 518	7.334 \pm 405	p < 0.001	NS

* SE: standard error

** NS: not significant

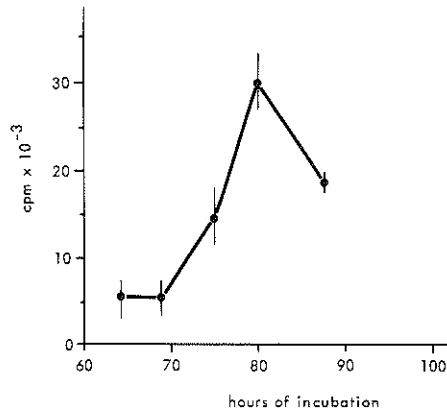


Figure 5:
Optimal incubation time of cultures in the LS-assay

¹⁴C-thymidine incorporation of pooled spleen cells from three immunized BALB/c mice after varying incubation times, in the presence of 1 µg MTV. The RLV control value was 5.3×10^{-3} cpm. Vertical bars indicate SE of triplicate cultures. Shown in the figure are the results of one out of four experiments. The results of all experiments were similar.

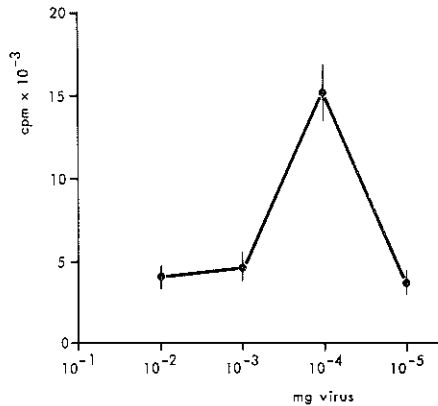


Figure 6:
Optimal MTV-concentration in the LS assay

¹⁴C-thymidine incorporation of pooled spleen cells from three immunized BALB/c mice in the presence of varying concentrations of MTV. Incubation time was 80 h. The RLV control value was 4.8×10^{-3} cpm. Vertical bars indicate SE of triplicate cultures. Shown in the figure are the results of one out of three experiments. The results of the three experiments were similar.

Testing for blocking and enhancing properties of serum.

The influence of serum was established by adding 0.1 ml test serum to the cultures; normal serum served as a control. MTV was added to both test and control cultures. Inhibition by serum factors was expressed as a percentage of the values of normal serum according to the formula $(1 - TS/NS) \times 100 \%$ in which TS is the average cpm of the cultures containing test serum, and NS represents that of the cultures containing normal serum. Also per cent inhibition by other suppressive agents (Chapter V) was calculated in this way.

2.6. Separation and testing of spleen cell subpopulations

Macrophages were removed by adherence of spleen cells (4×10^6 /ml) to the surface of Falcon tissue culture flasks (25 cm^2) during 2 - 3 h, or with the iron-magnet method. B cell-enriched fractions were obtained by incubating macrophage-depleted spleen cells with mouse anti-theta serum and complement for 1 h. The anti-theta serum was made by injecting 2×10^7 thymocytes derived from C3H mice ip into AKR mice. This was done six times at intervals of 14 days. Then the AKR mice were bled; the resulting anti-theta serum killed 70 % of C3H thymocytes up to a dilution of 1/16. As a source of complement, guinea pig serum absorbed with agarose and stored in liquid nitrogen was used. The complement was active up to a dilution of 1/8. After treatment the cells were washed and used for testing.

T cell-enriched populations from spleens were obtained by passage over a nylon wool column (Julius et al., 1973). After washing with medium about 20 % of the original cell population was recovered. Sixty - 88 % of the eluted cell population could be killed with mouse anti-theta serum and complement, whereas 3 - 10 % were Ig-positive in membrane-immunofluorescence studies (tested 6 times).

2.7. Detection of MTV and mouse anti-MTV antibodies

Membrane immunofluorescence. This method was mostly used to detect mouse anti-MTV-antibodies. Unless mentioned otherwise, the MTV positive AR 963 cell line that was developed from a C3H mammary tumour (Van Pelt, et al., 1976) was used as a target cell. After trypsinization, the cells were dispersed in Falcon Micro Titer 3034 plates with a Hamilton syringe (Sorg, 1974) (Hamilton Co., Whittier, Calif.). After a 45 min incubation with the test serum, the plates were washed three times in medium. Thereafter, they were incubated for 30 min with goat antiserum directed against mouse Ig, conjugated with fluorescein isothiocyanate (FITC) (Nordic Immunodiagnosics, Tilburg, The Netherlands) in a dilution of 1 : 20 in PBS. After three washings in medium, the cells were examined with an inverted Leitz Biovert microscope (E. Leitz, Inc., New York, N.Y.) equipped for epi-illumination with the following filter combinations: excitation, 2 x KP 490 + 1 mm Gg 455; emission, K 515 + KP 560. A 50 x /1.0 water-immersion objective and 6.3 x oculars were used. The sera were tested in two log dilutions. All sera were tested two times. Cells incubated with normal mouse serum and goat anti-mouse IgG-FITC, and cells incubated with PBS and the conjugate served as negative controls. Cells, incubated with positive mouse serum, and cells incubated with goat anti-MTV serum and subsequently with goat anti-goat IgG-FITC served as positive controls.

Lymphoid cells that were tested for the presence of Ig, theta-antigen, anti-MTV antibodies or MTV on the cell membrane were, except for the use of different antisera, treated in the same way.

Sepharose bead immunofluorescence assay. The method developed for the assay of immunoglobulins (Haaijman and Brinkhof, 1977) has been adapted to the detection of MTV

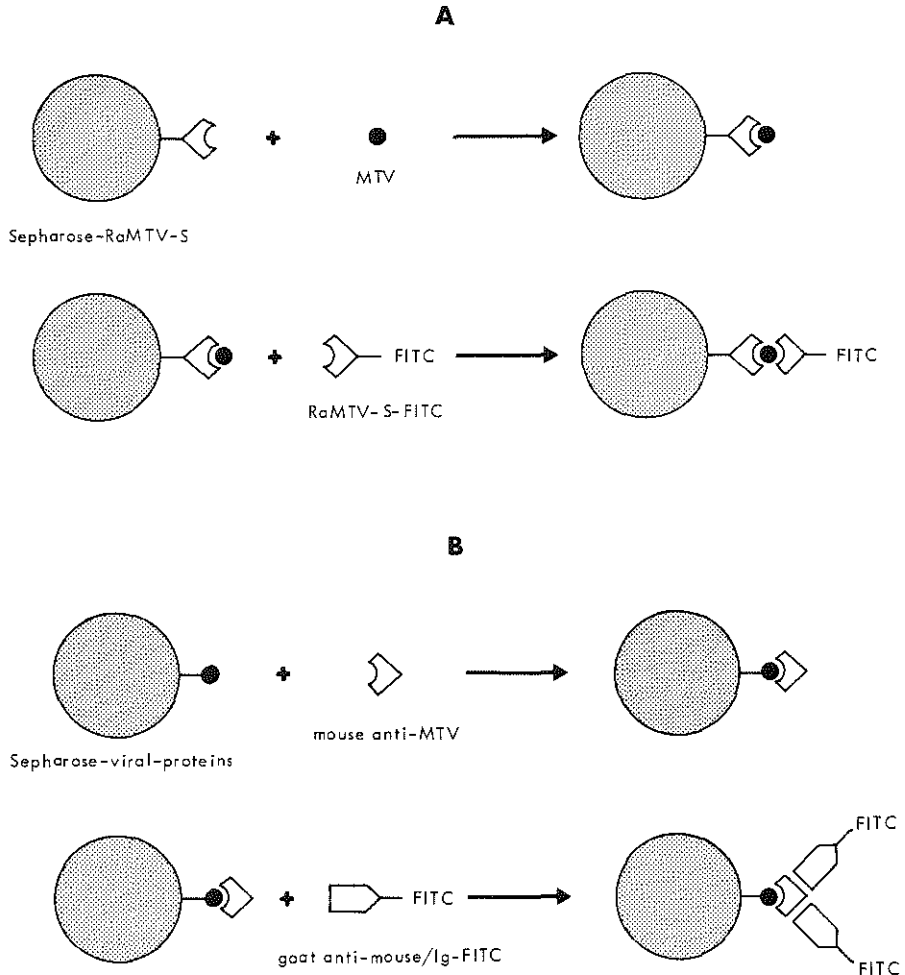


Figure 7:
Detection of MTV and anti-MTV antibodies by the Sepharose-bead immunofluorescence assay.

A: Detection of MTV. Rabbit anti-MTV-S serum (RaMTV-S) is coupled to Sepharose beads. After incubation with the sample to be tested, MTV is attached to the rabbit anti-MTV-S serum. MTV is then detected by incubation with rabbit anti-MTV-S IgG coupled to FITC (RaMTV-S-FITC).

B: Detection of mouse anti-MTV antibodies. MTV is coupled to Sepharose beads. After incubation with the serum to be tested, mouse anti-MTV antibodies are attached to MTV; the mouse anti-MTV antibodies are then detected by incubation with goat anti-mouse Ig coupled to FITC.

antigens (Haaijman, 1977). A rabbit antiserum to MTV was subjected to chromatography on QAE-Sephadex A50 (Pharmacia, Uppsala, Sweden) in ethanol-diamineacetate (pH 8.0; I 0.1), according to Joustra and Lundgren (1969). The isolated IgG fraction was dialyzed against PBS. To obtain a specific anti-MTV fraction, the IgG was then passed over an immunoadsorbent column, prepared according to Radl et al. (1974) consisting of immobilized normal mouse serum and an MTV-negative BALB/c mammary gland extract. Part of the unbound IgG was then coupled covalently to Sepharose 4-B beads (Pharmacia) by the cyanogen bromide method of March et al. (1974), using 2 mg protein per ml of activated beads. Residual active groups were deactivated by treatment of the beads for 6 h with 0.5 M ethanolamine, pH 9.5. The beads were then washed and stored in PBS with 0.01 % merthiolate. Another part of the IgG fraction was conjugated with FITC according to Hijmans et al. (1969).

For the determination of viral antigens, 50 μ l of a suspension of anti-MTV beads was incubated for 1 h with 50 μ l of a cell-free sample diluted in PBS containing 2 % bovine haemoglobin, in microtitration plates with continuous agitation. Control beads were incubated with PBS. After repeated washing, the beads were incubated with the FITC-conjugated antiserum to MTV (figure 7A).

After repeated washing the individual bead fluorescence was measured with a microfluorometer. At least five beads were measured per dilution.

For the detection of antiviral antibodies, beads coupled with MTV were used. Disrupted MTV was passed over a column consisting of a goat antiserum to normal mouse serum, coupled to Sepharose 4B as described above. Thereafter, the MTV preparation from which most host components were removed, was coupled to Sepharose beads as described above.

The beads were then incubated for 1 h with the samples, and then after repeated washing incubated with a goat antiserum directed against mouse immunoglobulins (figure 7B).

Beads coupled with normal BALB/c mouse serum served as a control.

2.8. Vaccination experiments

Preparation of vaccine. After ultrasonic disruption of the MTV-virions, host proteins, which constituted approximately 20 % of the total protein content of the virus preparation, were removed by absorption with Sepharose 4B beads covalently coupled with MTV-negative goat antimouse mammary gland serum. This virus protein fraction was chromatographed over a Con A-Sepharose 4B column. Bound glycoproteins were eluted with 0.2 M methyl- α -D-mannopyranoside in PBS. The eluted proteins were thereafter dialyzed against PBS (pH 7.2) for 4 days. The proteins in the fraction were identified by 0.1 % SDS (pH 7.0) - PAGE (Weber and Osborn, 1969). The gels were scanned spectrophotometrically in a gel scanner at 540 nm (Figure 8). In the preparation are a number of contaminants derived from Con A. They have the following molecular weights: 75,000; 28,000; 18,000; and 14,000 D. This has been estimated by PAGE with the use of various reference molecules. The major part (60 %) of this protein preparation was gp52. The vaccines of disrupted virus used in the experiments were obtained by freeze-thawing ten times.

When no adjuvant was used, the gp52-enriched protein fraction was diluted in PBS for the vaccination. When virus or the gp52-enriched protein fraction was precipitated on alum, the proteins were dissolved in 0.25 ml PBS; after the addition of 0.11 ml 1 N bicarbonate solution, 0.25 ml of a 10 % alum solution was added. The solution was left overnight at 4^o C, and the alum precipitate was then pelleted by centrifugation at 3,000 rpm for 30 min. No proteins could be detected in the supernatant. The precipitate was afterwards dissolved in PBS to the desired concentration.

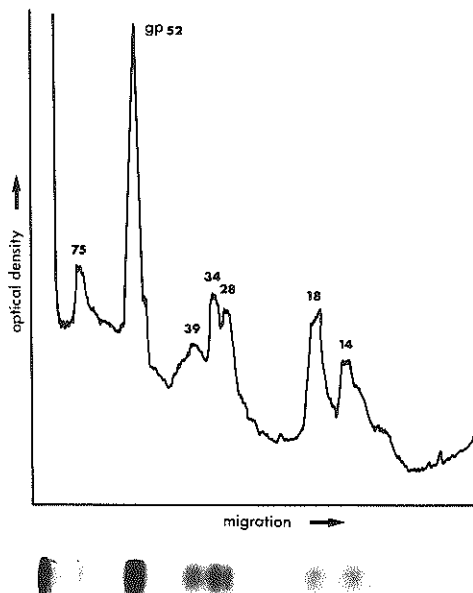


Figure 8:
 Identification of polypeptides in the MTV-S protein vaccine

SDS 0.1 % (pH 7.0) - 10 % polyacrylamide gel electrophoretic analysis of the MTV-S polypeptide fraction eluted from a Con A-Sepharose 4B column. The protein fraction was dissociated at 100° C for 2 min in the presence of 1 % SDS and 1 % β -mercapto-ethanol. Electrophoresis was done for 18 h at 3 mA/gel. The gel was stained with Coomassie blue and scanned at 540 nm.

When virus or the gp52-enriched protein fraction was used in combination with IPM, both proteins were dissolved in PBS. The IPM was provided by Dr. Lamensans, Institute Pasteur, Paris, France.

Challenge with tumour cells. For the study of trans-plantation resistance, vaccinated mice were challenged with their syngeneic tumour: BALB/c mice were injected with an MTV-O induced tumour, GR mice with an MTV-P induced tumour and the DBAf mice with the L1210 leukaemia. Before challenge, the solid mammary tumours were forced through a fine steel mesh; after washing (3 times 26 x g for 10 min) in medium, the cells were drawn through a fine-gauge needle. The viable cells were then counted and injected subcutane-

ously (sc) into the mice.

The L1210 leukaemia was maintained alternately *in vivo* and *in vitro* for periods of 2 weeks. The cells were maintained *in vitro* in plastic Falcon culture flasks (Flow Laboratories, Irvine, Scotland) and *in vivo* by ip injection into (DBAf x BALB/c)F1 mice. Before challenge, the cells were washed once and drawn through a fine gauge needle; afterwards, they were injected ip into the mice.

The challenge dose varied (see Chapter VI).

Experimental design. To test transplantation resistance in BALB/c and DBAf mice, different doses of vaccines were injected ip into the animals. Controls were treated with the adjuvant alone. Controls and vaccinated animals were challenged simultaneously with tumour cells 20 days after vaccination. To investigate the influence of the adjuvants, a group of untreated animals was challenged with tumour cells. After challenge with a mammary tumour, the animals were examined every 3 days; they were killed as soon as a tumour was observed. The experiments were terminated 30 weeks after challenge; animals that had developed no tumour by then were considered as survivors. When the animals were challenged with leukaemia cells, death was taken as a parameter for tumour incidence. Deaths were recorded two times a day; if the animals had not died at 5 weeks after challenge, they were scored as survivors. Statistical differences of latency periods between vaccinated and control animals were established by the two-tailed Students't-test.

At several intervals after vaccination, 2 animals from each group were bled from the retro-orbital plexus and, after killing by cervical dislocation, the combined peritoneal cells were taken for the *in vitro* studies on immunity. The sera were also pooled.

In the experiments on vaccination against primary tumour development, female mice were inoculated s.c. with the vaccine at 8 - 16 weeks of age; at the age of 22 - 37 weeks, they were subjected to forced breeding. The mice were examined twice a week for the presence of tumours. They were killed immediately when such signs were present. Animals, that did not develop a mammary tumour were permitted to live out their lifespan.

Cellular immune reactivity was, besides by means of the direct LAI assay, also investigated with the Winn-assay. For this test, five thousand syngeneic mammary tumour cells suspended in RPMI 1640 medium were mixed with 5 or 25 x 10⁶ immune spleen cells and injected sc into syngeneic 6-week-old mice. Control animals were injected with the same mixture of normal spleen cells and tumour cells (Winn, 1961). The grafted mice were examined every three days for tumour development. The observation period was five months.



CHAPTER III

MTV-DIRECTED IMMUNITY IN MICE DURING THE GROWTH OF SPONTANEOUS TUMOURS

Although healthy mice show immunologic responses against the antigens of MTV-induced tumours, this response is unable to prevent tumour development. After development of a mammary tumour the anti-MTV responses become even higher (Chapter I: 3,4). It may therefore be concluded that MTV antigens are immunogenic. Nevertheless, this immune response is inadequate. Just before the tumour becomes clinically detectable, antigen levels in the blood of mice increase sharply (Ritzi et al., 1976). The possibility exists that antigen in the circulation inhibits the immune effector arm.

Since it is a very complicated procedure to study mice that are just about to develop a tumour, we decided to investigate mainly the anti-MTV responses in mice bearing spontaneous mammary tumours of varying sizes. We investigated animals of the GR, the BALB/c and the BALB/cfC3H strain; tumour development in these mice was stimulated by forced breeding. The presence of anti-MTV antibodies in sera from individual tumour bearing mice was established by membrane immunofluorescence on living tumour cells. Cellular immunity was investigated with both the direct LAI and the LS assays. Individual mice were tested with the LAI assay; LS tests were performed with pooled cells from three mice bearing tumours of approximately the same size. MTV-S was used as an antigen in both tests.

3.1. Membrane immunofluorescence

The presence of anti-MTV antibodies was established by means of membrane immunofluorescence. We tested undiluted

Table 7

MTV-ASSOCIATED* MEMBRANE IMMUNOFLUORESCENCE WITH SERA FROM TUMOUR
BEARING BALB/c MICE

Mouse strain	serum donors		fluorescence on of cultures G ⁻ -MTC**	
	MTV-strain	tumour weight (g)	45 min	16 h
BALB/c	MTV-O	2.0	-	+
		3.2	-	+
		4.0	-	±
BALB/cfC3H	MTV-S	0.7	-	-
		1.3	+	+
		1.8	++	++
		2.3	++	++
		5.0	-	-

* All sera revealed negative results when tested on BALB/c derived embryonic fibroblasts.

** GR-MTC: GR-derived mammary tumour cells

mouse sera, since dilution rapidly led to the disappearance of positive fluorescence. In addition to the commonly used serum incubation time of 3/4 h, we also tested fluorescence after an incubation period of 16 h. Since the anti-MTV antibodies in mice that are induced by different MTV variants also react to the group-specific antigens of MTV (Ihle et al., 1976), the sera from the BALB/c strains were tested against GR-derived mammary tumour cells. BALB/c-derived embryonic fibroblasts served as a specificity control.

The results are summarized in table 7. From these results it is obvious that tumour bearing BALB/c and BALB/cf C3H mice have antibodies in their serum which give a broad cross-reactive membrane fluorescence with GR mammary tumour cells producing MTV-P. Mice which were infected with MTV-S along with the endogenous MTV-O showed a much stronger reaction than mice infected with MTV-O only: the latter sera gave positive results only after the prolonged incubation period of 16 h. Absorption of one positive BALB/c and one positive BALB/cfC3H serum with purified MTV-S led to abrogation of most, but not all fluorescence. When the sera were incubated for 3/4 or 16 h on BALB/c embryonic fibroblasts, no fluorescence was observed. These results indicate that the antibody concentration varies between animals carrying tumours of different sizes.

To investigate if there is any relation between antibody level and tumour growth, we tested the sera from three individual tumour bearing GR mice over a period of 3 months at intervals of two weeks. The bleeding was started when no tumour could be detected. Twice a week the animals were searched for tumours. The sera obtained from the last bleeding before the tumour became clinically detectable, and from all following bleedings were used for the membrane immunofluorescence studies. Primary cultures of GR-derived mammary tumour cells were used as target cells, GR-derived embryonic fibroblasts served as a specificity control. Incubation time of sera was 3/4 h. All sera from one animal were tested simultaneously. The results are summarized in

Table 8

MEMBRANE IMMUNOFLOURESCENCE WITH SERA TAKEN FROM GR MICE IN THE COURSE OF
TUMOUR DEVELOPMENT

day of testing	fluorescence on cultures of GR-derived mammary tumour cells*		
	mouse 1	mouse 2	mouse 3
1**	-	+	NT***
13	-	+	-
27	+++	-	-
43	+++	-	-
51	++	++	+++
65	-	++	++
78	++	-	+

* All sera yielded negative results when tested against GR-derived embryonic fibroblasts.

** day one refers to the date of the last bleeding before the tumour became clinically detectable.

*** NT: not tested.

table 8. They clearly show that antibody concentration in GR mice oscillates during tumour growth; antibodies are sometimes even absent.

3.2. Direct LAI assay

When peritoneal cells derived from individual BALB/c, BALB/cfC3H and GR mice bearing primary tumours were tested in the direct LAI-assay, a significant difference in the number of adherent cells between the wells with MTV and RLV was found in 18 out of 42 animals tested.

During the experiments with individual mice bearing tumours, it was constantly observed that reactivity was highest when the tumour was small (varying from 0.5 to 1.7 g); with increasing tumour weight, reactivity disappeared, whereas animals bearing very large tumours (above 3 to 4 g) again showed some reactivity. Since differences in the agents used may exert some influence on the reactivity measured, individual BALB/c, BALB/cfC3H and GR mice bearing primary tumours of varying size were tested with the same antigen preparation and batch of FCS. The results are shown in fig. 9.

It was found that reactivity was stronger in BALB/cfC3H mice than in the other two strains. Therefore it was investigated whether type-specificity of the MTV strain that is used as the antigen influences the results obtained in the LAI-assay. Inhibition of adherence of peritoneal cells from GR mice after incubation with the autologous MTV strain (MTV-S) and with homologous MTV-P was tested. The results, summarized on table 9, indicate that MTV-specific cellular immune reactivity as measured with the LAI assay is not dependent on type-specificity.

Table 9

DIRECT LEUKOCYTE ADHERENCE INHIBITION IN TUMOUR BEARING GR MICE UPON INCUBATION WITH DIFFERENT
MTV STRAINS

Exp. no.	Incubation with RLV	Incubation with MTV-S	Incubation with MTV-P
	Average no. of adherent cells \pm SE*		
1	14.4 \pm 2.1	8.0 \pm 1.6	6.5 \pm 0.9
2	86.8 \pm 5.8	49.2 \pm 3.4	45.7 \pm 3.9
3	27.6 \pm 1.5	25.1 \pm 1.0	21.4 \pm 3.0
4	42.0 \pm 3.2	28.2 \pm 2.1	24.1 \pm 3.2

* SE: standard error

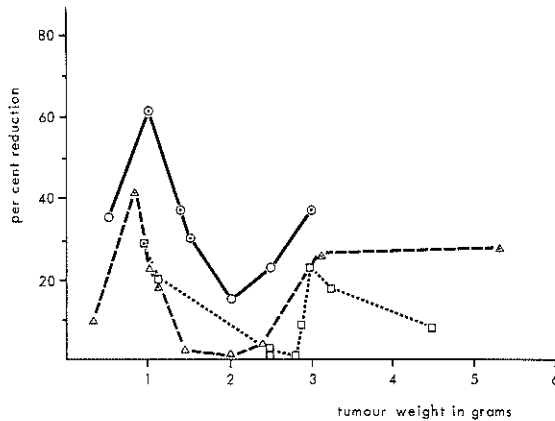


Figure 9:
 MTV-specific LAI-reactivity of peritoneal cells from tumour-bearing mice

Individual mice bearing primary tumours of varying size were tested by means of the direct LAI-assay. All tests were performed with the same MTV-S antigen preparation and the same batch of FCS.

○ : BALB/cfC3H mice; □ GR mice; △ BALB/c mice.

A dot in the symbols indicates significant ($p < 0.025$) reduction of adherence as compared to the RLV control value.

3.3. LS test

In this assay we tested pools of cells derived from mice bearing tumours of approximately the same size. The spleen, the axillary and the inguinale lymph nodes were removed; the isolated cells were pooled prior to testing. Spleen cells and lymph node cells were tested separately.

In 29 out of 62 tests performed with cells from tumour bearing animals, a reaction specific for MTV as compared to RLV was observed. The ^{14}C -thymidine uptake was 2 - 4 times that of the control.

In this assay, the fluctuation in response also seemed to be related to tumour size. In fig. 10 are plotted the values for MTV-specific stimulation against tumour weight. The data were obtained in a very short period of time (1 month). To keep conditions as standardized as possible, the same antigen preparations and FCS batch were used. A simi-

Table 10

¹⁴C-THYMIDINE INCORPORATION OF SPLEEN CELLS FROM TUMOUR BEARING MICE UPON INCUBATION WITH DIFFERENT MTV STRAINS

Mouse strain	MTV strain	Incubation with RLV	Incubation with homologous MTV-S cpm ± SE*	Incubation with Autologous MTV
BALB/c	MTV-O	3.003 ± 938	6.242 ± 1.038	6.276 ± 1.234
		2.403 ± 273	4.881 ± 139	5.029 ± 460
GR	MTV-P	1.751 ± 714	3.743 ± 324	3.380 ± 138

* SE: standard error

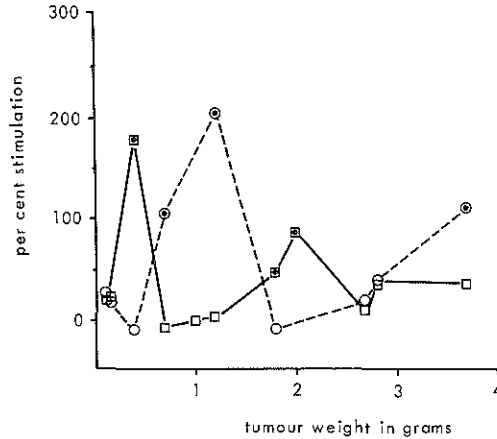


Figure 10:
 MTV-specific ^{14}C -thymidine-incorporation of spleen and lymph node cells
 from tumour-bearing BALB/cfC3H mice

The tests were performed with pools of cells derived from three animals bearing tumours of approximately the same size. For all tests, the same MTV-S antigen preparation and batch of FCS were used.

□: spleen cells; ○: lymph node cells.

A dot in the symbols indicates significant ($p < 0.025$) stimulation as compared to the RLV control value.

lar fluctuation related to tumour size as was found with the LAI assay is observed. It is remarkable that the results for spleen cells do not parallel those obtained with lymph node cells. In various other experiments, we noticed the same general pattern: peak reactivity when tumour weight is small, a steep decline with increasing tumour weight and partial recovery when tumour size is very large.

The specific response to MTV is not dependent on the virus strain that is used as antigen. A representative experiment is shown in table 10; repeated tests yielded similar results.

3.4. Discussion

Tumour bearing BALB/c and BALB/cfC3H mice express antibodies against GR tumour cell membranes; since this reaction is partly inhibited by adsorption with MTV-S, anti-MTV antibodies are likely to be present. This is in accord with previous findings (Bentvelzen et al., 1970; Ihle et al., 1976). It was remarkable that some sera showed positive fluorescence only after a prolonged incubation period of 16 h; this might indicate that these sera contained only very low concentrations of antibody, or that the antibody was bound to antigen and that the resulting complexes had only a very weak affinity for the target cells.

The results with the individual tumour bearing GR mice clearly show that the antibody content fluctuates in this strain; the results from the BALB/c mice bearing MTV-O and MTV-S induced tumours indicate that the antibody content in this strain during tumour growth is also not stable. Reactivity was stronger in the BALB/cfC3H strain than in BALB/c mice; this may be due to the fact that virions of the different MTV strains show some serological differences, as detected by heterologous antisera (Daams et al., 1973; Teramoto et al., 1977).

With two different techniques, the LAI and the LS tests, it was found that mice bearing mammary tumours have cellular immunity to MTV. Specificity of the reaction was mainly deduced from significant differences in reactivity of the peritoneal cells when relatively small amounts of MTV were added as compared to the addition of similar amounts of an unrelated oncovirus (RLV) or ovalbumin.

The stimulation of cell proliferation as measured by ^{14}C -thymidine uptake upon incubation with MTV is relatively low (2-4 times the control value) as compared to stimulation by plant lectins. Similar low values have been found in the mixed leukocyte-tumour cell interaction test for a virally induced rat lymphoma (Glaser et al., 1975).

It is remarkable that very often when the spleen cells of tumour-bearing animals were reactive in the LS-assay, the lymph node cells from the same animals did not respond, or *vice versa*. This phenomenon has also been observed in the microcytotoxicity test for the B16 mouse melanoma (Bartholomeus et al., 1974).

In the LAI and LS tests, the anti-MTV reactivity seems to be mainly evoked by group-specific antigens. In the present study, the results of LAI and the LS assays seem to coincide; it must be mentioned however, that cells from the same animals have not been compared in both tests.

In both tests, the highest reactivity was found when the animals had small tumours. This indicates that a relation exists between cellular immunological reactivity and antigenic load, although tumour weight is only a rough estimate of the latter. From other animal studies (Rowland et al., 1972; Blair, 1976; Glaser et al., 1975) it is also known that cellular immunological reactivity is generally diminished with increasing tumour size. Decreased reactivity against tumour specific antigens, as revealed by reduced transplantation resistance (Vaage, 1973), may be associated with the general immunological impairment.

After the initial peak in reactivity at low tumour weight, an almost complete loss of activity was found; however, in animals with very large tumours (above 3 g), the activity reappeared again, but at a lower level than was found with cells from animals with small tumours. Thus, cellular reactivity fluctuates. For opsonins, also an oscillation during tumour growth has been observed (Saba and Antikatzides, 1975).

An explanation for the decrease in cellular responsiveness to MTV in both tests may be the saturation of receptors of immunocytes with increasing amounts of viral antigens that are released into the blood stream when the tumour progresses (Moore et al., 1970). A second possibility is the oc-

currence of suppressor cells which inhibit the response of the otherwise reactive cells to MTV. Both immune suppressive mechanisms will be dealt with in Chapter V.

CHAPTER IV

THE ROLE OF SPLEEN CELL SUBPOPULATIONS IN MTV-DIRECTED CELLULAR IMMUNITY

To obtain more insight into the suppressive factors that are responsible for the depression in MTV-directed cellular immune reactivity that occurs when tumour growth progresses, it is necessary to first establish which immunocytes react against MTV.

For this purpose spleen cells from immunized DBA/1 mice were treated with crude cell separation techniques (Chapter II;6). The MTV-directed reactivity of the spleen cell subpopulations was then established with the LS and the LAI-assay. For these studies, the indirect variant of the LAI-assay was employed, since a stepwise analysis of the *in vitro* immunological events is much easier to realize in this test than in the direct LAI-assay (Chapter II;3,4).

Evidence is presented that the LS and LAI assays are both T cell dependent. It is well-known that macrophages are required for mitogen-induced T cell proliferation (Rosenstreich et al., 1976); in this Chapter, we demonstrate that macrophage-like cells also play a crucial role in the LAI assay.

4.1. Mechanisms of MTV-induced LAIF production

The role of different cell subpopulations. As mentioned in Chapter II, Holt et al. (1975) reported that the antigen-induced inhibition of adherence of leukocytes can be transferred to normal adherent cells by a soluble factor, LAIF, that is present in the supernatant of immune spleen cells incubated with the antigen. Our indirect test is based on this finding. According to the observations of

Table 11

MTV-SPECIFIC LAIF PRODUCTION OF DIFFERENT CELL SUBPOPULATIONS PREPARED FROM IMMUNE
SPLEEN CELLS AS MEASURED BY THE INDIRECT LAI ASSAY

Cell subpopulation	Exp. 1		Exp.1	Exp.2	Exp.3
	Incubation with RLV	Incubation with MTV			
	Average no. of adherent cells \pm SE ^a		Reduction of adherence (%)		
Spleen cells	71.3 \pm 3.7	49.6 \pm 3.3	30*	35*	25*
Macrophage-depleted spleen cells	44.6 \pm 1.6	43.2 \pm 1.4	3	2	3
B cell-enriched ^b	43.8 \pm 2.8	44.6 \pm 3.0	0	1	0
T cell-enriched ^b	27.9 \pm 2.5	29.6 \pm 1.9	0	2	0
T cell-enriched and adherent cells	55.3 \pm 2.3	38.3 \pm 1.7	31*	27*	26*

^a SE: standard error

^b see text, Chapter II:6

* significant reduction of adherence ($p < 0.001$)

Holt, the indirect LAI reaction can be abolished by pretreatment of the spleen cells with anti-theta-serum and complement.

For this study, we used in each experiment pooled spleen cells obtained from at least 5 immunized DBA/1 mice. As antigen MTV-S, purified as described in Chapter II.2, was used. In each experiment, the RLV control cells received the same treatment as the cells incubated with MTV.

First, we established which cell subpopulations are necessary for MTV-specific LAIF-production. T-cell enriched fractions were obtained by passage over a nylon wool column. Adherent cells were removed by plastic adherence; the adherent cells present in 20×10^6 spleen cells were allowed to attach to the surface of the plastic tubes in which the test was performed. After washing medium was added. When adherent cells in combination with T cell enriched cell fractions were tested, adherent cells were obtained in the same way; instead of medium, the T cell-enriched fraction was added. B-cell enriched fractions were obtained by first removing the adherent cells; then the cell suspension was treated with anti-theta serum and complement. The cell subpopulations were then tested simultaneously.

The results, summarized in table 11 show that LAIF production could be inhibited by pretreatment of the spleen cells with mouse anti-theta serum and complement. Negative results were also obtained with T cell-enriched fractions, and with spleen cell populations depleted of adherent cells. If a T cell-enriched fraction was added to adherent cells, a positive result was obtained.

To determine whether direct cell-to-cell contact between T cell-enriched fractions and adherent cells is necessary for LAIF production, these cell populations were cultured so that exchange of soluble factors could take place, while the cells were separated by a Millipore filter: Adherent cells were allowed to attach to the bottom of plastic tubes (Falcon, 75 x 100 mm). Afterwards, sterilized

Table 12

MTV-SPECIFIC LAIF PRODUCTION BY IMMUNE T CELL-ENRICHED CELL POPULATIONS AND ADHERENT CELLS WHEN CULTURED IN THE SAME MEDIUM BUT SEPARATED BY A MILLIPORE FILTER

Culture method	Exp. 1		Exp.1	Exp.2	Exp.3
	Incubation with RLV	Incubation with MTV			
	Average no. of adherent cells \pm SE*		Reduction of adherence (%)**		
Cell populations mixed	21.2 \pm 0.9	16.1 \pm 0.5	24	31	37
Cell populations separated by a Millipore filter	21.1 \pm 1.0	15.0 \pm 0.4	29	34	38

* SE: standard error

**all values were significant ($p < 0.001$)

Millipore filters with a diameter of 47 mm and a pore size of 0.45 μ (Millipore, Bedford, Mass.) were moistened in medium and pushed into the tubes in such a way that they became funnel-shaped. Only the point of the filter made contact with the underlying medium. One ml of a T cell enriched fraction was then transferred into the funnel-shaped filter. After the incubation period, the filters were removed and the remaining cells were spun down; then the supernatant was tested.

The results, summarized in table 12, demonstrate that LAIF production is not decreased if the cells are separated by a Millipore filter. Therefore, cell-to-cell communication, mediated by soluble factors, is likely to trigger LAIF-production.

To study the sequence of interactions between T cells and adherent cells, supernatant first incubated for 24 h with MTV and adherent cell cultures was added to a T cell-enriched fraction. After another 24 h incubation period the supernatant was tested for LAIF-production. Also the reverse sequence of incubation (i.e. supernatant incubated with T cell enriched fractions transferred to adherent cells) was tested. Prior to adding a supernatant to a different cell population, the cells were washed two times. Surprisingly, both sequences of incubation yielded negative results. For this reason, the incubation scheme was extended: MTV was added to adherent cells; after a 24 h incubation period, the supernatant was transferred to a T cell enriched fraction, and after another 24 h period of incubation transferred to adherent cells again. After incubation, the resulting supernatant was tested for LAIF-production. Also the sequence of transfer T cell enriched - adherent cells - T cell enriched was tested. The RLV control supernatants were prepared in the same way.

The results are presented in table 13. All combinations shown on this table were tested simultaneously. The results clearly show that inhibition of adherence is achieved only when the following order of transfer was

Table 13

EFFECT OF DIFFERENT SUPERNATANTS ON MTV-SPECIFIC IMMUNE ADHERENCE^a.

Production of supernatants by	Exp. 1		Exp.1	Exp.2	Exp.3
	Incubation with RLV Average no. of cells \pm SE ^b	Incubation with MTV Average no. of adherent cells \pm SE ^b	Reduction of adherence (%)		
T ^c + A ^d	88.9 \pm 1.7	57.1 \pm 3.7	36* ^e	40*	35*
T	37.2 \pm 1.9	37.0 \pm 1.4	0	0	2
A	38.7 \pm 3.3	37.4 \pm 1.0	3	1	4
T \xrightarrow{f} A	70.6 \pm 1.3	71.8 \pm 1.7	0	3	2
A \rightarrow T	80.5 \pm 2.4	83.8 \pm 2.1	0	0	1
T \rightarrow A \rightarrow T	82.6 \pm 5.4	81.2 \pm 3.9	2	4	6
A \rightarrow T \rightarrow A	110.0 \pm 5.2	58.5 \pm 4.1	47*	31*	45*

a incubation period with each cell subpopulation was 24 h

b SE: standard error

c T : T cell enriched subpopulations (Chapter II.6)

d A : adherent cells

e * : $p < 0.001$

f \rightarrow : arrow indicates "supernatant transferred to"; the final supernatant was transferred to indicator cells.

used: adherent cells - T cells - adherent cells.

The role of antigen. It is interesting to know whether antigen continues to play a role after the initial incubation with adherent cells, or whether cell-to-cell communication is exclusively mediated by the soluble factors produced by adherent cells and the T cells, referred to as SF₁ and SF₂, respectively. For this reason, the MTV was removed from the SF₁-containing medium by treatment with rabbit anti-MTV serum bound to Sepharose beads.

For sterilization, these beads were left overnight at 4° C in a 2 % sodium azide solution in PBS, and then washed 4 times in medium. After this treatment, the beads were still active in binding MTV, as was shown by the Sepharose bead immunofluorescence technique. The number of beads added to 1 ml of supernatant could bind approximately 100 µg of MTV. After the incubation period, the beads were spun down and the supernatant was transferred to T cells and then to adherent cells again prior to testing.

The results, summarized in table 14, show that LAIF production induced by SF₁ is not decreased when the antigen is removed after the initial incubation of the adherent cells with MTV. Control experiments in which MTV was removed with the adsorbent prior to adding the medium to immune adherent cells showed that the production of SF₁ was completely prevented.

4.2. MTV-specific proliferating cells in the LS assay

To investigate which cell subpopulation reacted with proliferation in the LS assay upon incubation with MTV, ¹⁴C-thymidine incorporation of different spleen cell-subfractions after incubation with MTV was tested. Spleen cells were taken from immunized BALB/cfC3H and GR mice; MTV-S (Chapter II;2) was used as the test antigen.

Table 14

INFLUENCE OF REMOVING MTV FROM THE SF₁-CONTAINING SUPERNATANT ON THE
RESULTING MTV-SPECIFIC LAIF PRODUCTION

Exp. no.	SF ₁ depleted of MTV		Untreated SF ₁	
	Average no. of adherent cells \pm SE**	Reduction of adherence (%)	Average no. of adherent cells \pm SE	Reduction of adherence (%)
1	45.8 \pm 4.0	45	32.5 \pm 6.8	61
2	66.2 \pm 3.7	47	69.5 \pm 4.0	45
3	53.8 \pm 5.0	42	59.8 \pm 3.2	36

* Reduction as compared to RLV control; all values were significant (p < 0.001)

** SE: standard error

Table 15

MTV-SPECIFIC ^{14}C -THYMIDINE UPTAKE OF VARIOUS SPLEEN CELL SUBPOPULATIONS OF IMMUNIZED MICE

Mouse strain	Cell population	Experiment 1		Exp. 1	Exp. 2	Exp. 3
		Incubation with RLV cpm \pm SE ^a	Incubation with MTV cpm \pm SE	Stimulation (%)		
BALB/cfC3H	Lymphocytes ^b	2,237 \pm 712	3,226 \pm 233	44	121*	87*
	T-cell-enriched ^c	1,652 \pm 318	3,595 \pm 184	117* ^d	246*	154*
	B-cell-enriched ^c	1,086 \pm 312	1,224 \pm 218	13	29	0
GR	Lymphocytes	5,161 \pm 561	15,889 \pm 249	211*	53	74*
	T-cell-enriched	1,721 \pm 203	7,273 \pm 567	322*	96*	137*
	B-cell-enriched	2,640 \pm 416	2,841 \pm 268	17	4	2

^a SE: standard error^b spleen cells depleted from adherent cells^c Chapter II.6^d *statistically significant ($p < 0.010$)

Table 16

¹⁴C-THYMIDINE INCORPORATION OF T CELL-ENRICHED SPLEEN CELL SUBPOPULATIONS OF IMMUNIZED MICE, CULTURED WITH IMMUNE OR NORMAL ADHERENT CELLS

Mouse strain	Cell population	Experiment 1		Exp.1	Exp.2
		Incubation with RLV cpm ± SE ^a	Incubation with MTV cpm ± SE	stimulation (%)	
BALB/cfC3H	T cell-enriched fractions ^b and immune adherent cells	1,991 ± 321	3,047 ± 207	50 ^c	98* ^d
	T cell-enriched fractions and normal adherent cells	2,173 ± 207	3,001 ± 57	46 ^e	74*
GR	T cell-enriched fractions and adherent cells	1,805 ± 134	3,698 ± 325	105*	145*
	T cell-enriched fractions and normal adherent cells	2,157 ± 288	4,025 ± 178	87*	136*

^a SE: standard error

^b Chapter II.6

^c not significant

^d *:p < 0.010

^e p < 0.025

To obtain comparable results, the MTV-specific ^{14}C -thymidine uptake of T cell enriched and B cell enriched spleen cell fractions was compared to the ^{14}C -thymidine uptake of spleen cells depleted from adherent cells (i.e. lymphocytes). The results, demonstrated on Table 15, indicate that the T cell-enriched fraction showed the highest proliferation as compared to the RLV-control, whereas the degree of proliferation observed with the same number of cells of the B cell-enriched population was negligible. Even if stimulation of the lymphocytes was not significant, significant stimulation emerged when T cell-enriched cell fractions were used.

When immune adherent cells were added to the T cell-enriched fraction proliferation was not increased, as compared to T cells to which normal adherent cells were added (Table 16).

4.3. Discussion

In the LAI-assay, pretreatment of the spleen cells with mouse anti-theta serum and complement completely abolishes the LAIF production, thereby confirming the results of Holt et al. (1975). However, a T cell-enriched fraction containing at least 60 % theta-positive cells also failed to induce the LAI-reaction, while a positive result was found when adherent cells were added. We therefore conclude that, at least in the MTV system, both T cells and adherent cells are necessary for producing LAIF. The adherent cells which can be removed by the iron-magnet method or by adherence to plastic surfaces may well be monocytes (Grosser et al., 1976).

Marti et al. (1976), who performed the LAI assay with peripheral blood lymphocytes from breast cancer patients, could not find any evidence for a soluble mediator that is responsible for the inhibition of adherence, nor could Holan et al. (1974), who used rat lymph node cells. A

possible explanation for these negative results is the source of lymphoid cells used: preliminary results indicate that in the indirect assay lymph node and peripheral blood cells of immunized animals do not show a positive LAIF-reaction, whereas spleen cells do. Probably, one subpopulation needed for the eventual release of LAIF is present in too low proportion in peripheral blood and lymph nodes.

From the experiments in which T cells and adherent cells were cultured in the same medium, while the cells themselves were separated by a Millipore filter, and from the supernatant transfer experiments it can be concluded that direct cell-to-cell contact is not required for LAIF production, but that interaction is mediated by soluble factors.

To detect which interactions between the adherent cells and the T cell enriched fractions had to take place for the production of LAIF, supernatant incubated with one of these cell fractions was transferred to another; the resulting supernatant was tested. Various sequences of incubation were tested. From our results, it became evident that LAIF production occurred when a) adherent cells were incubated with the antigen; b) the supernatant was transferred to T cell-enriched cell populations; and c) the supernatant of the latter was again transferred to adherent cells.

Our results suggest the following sequence of reactions: When antigen is presented to adherent cells, they release a soluble factor, which we shall call SF_1 . When SF_1 interacts with T cells, these produce another soluble factor, SF_2 . When SF_2 interacts with adherent cells, they release LAIF, which is responsible for the inhibition of adherence of leukocytes (fig. 11).

No significant decrease in LAIF production was found when antigen was removed from the SF_1 containing medium; thus it is concluded that, after the initial incubation with adherent cells, soluble antigen is not longer required. One must bear in mind that part of the Ig-positive B cells have adherent properties; moreover, blocking and

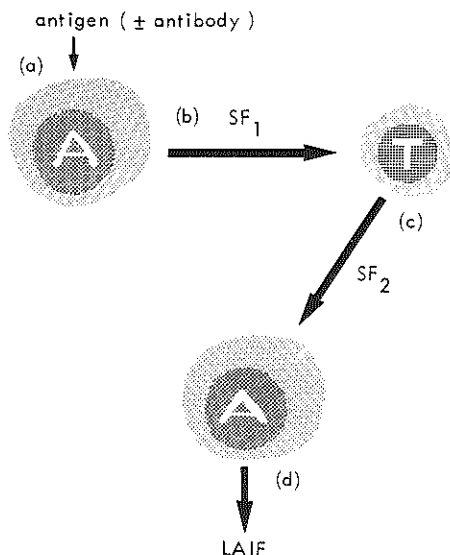


Figure 11: Mechanisms of LAIF production

(a) Antigen, whether or not complexed with antibody, contacts adherent cells; (b) upon this contact the adherent cells release a soluble factor, SF_1 , which reacts with T cells; (c) this event stimulates the T cells to release another soluble factor, SF_2 ; (d) SF_2 stimulates the adherent cells to produce LAIF, which inhibits the adherence of leukocytes.

enhancing properties of serum can be established with the LAI assay (Halliday and Miller, 1972). Thus it is possible that antibody produced *in vitro* by adherent B cells binds the added antigen; the ingestion of these complexes by macrophages may be the signal to produce SF_1 . It remains unclear whether antigen-antibody complexes are required for the release of the other soluble factors that are involved in this system.

Also in the LS assay it was analysed which spleen cell subpopulation reacted upon incubation with MTV. From the experiments on ^{14}C thymidine incorporation with the spleen cell subpopulations, it can be concluded that the MTV-specific reactivity in the LS assay observed in splenic cell cultures derived from BALB/cfC3H and GR mice immunized with MTV is due primarily to proliferation of T cells.

The addition of adherent cells derived from immunized animals did not enhance the proliferation of T cell-en-

riched cell fractions as compared to when normal adherent cells were added. It is therefore not likely that specifically primed macrophages are required in large quantities for the induction of specific T cell proliferation.

CHAPTER V

SUPPRESSIVE FACTORS INTERFERING WITH CELLULAR IMMUNE RESPONSES TO MTV IN TUMOUR BEARING MICE

Immune reactivity to MTV declines during tumour growth (Chapter III). Like mentioned in Chapter I.4, several authors have reported the existence of suppressor cells with adherent properties, which inhibit the aspecific cellular immune response. In addition, it has been found that the cellular immune response in leukaemic patients could be enhanced by slight trypsinization of the immunocytes. In many tumour systems, serum blocking factors that inhibit cellular immune reactivity have been established.

We have therefore investigated whether such factors interfere with the MTV-directed cellular immune reactivity in tumour bearing mice. During these studies we detected that the following immune suppressive mechanisms are present during tumour development: 1) suppressor cells with adherent properties that inhibit the MTV-specific proliferation of T lymphocytes; 2) a suppressive factor covering T lymphocytes (and probably macrophages) and thus making them unresponsive in both the LAI and LS assays; 3) serum factors inhibiting LS and LAI reactivity.

Evidence is presented that the latter two factors are probably antigen-antibody complexes.

5.1. Suppressor cells with adherent properties

To detect which cells influenced MTV-specific proliferation, T cell-enriched fractions from tumour bearers were tested in the LS assay in combination with adherent cells. For this purpose, the adherent cells present in 2.5×10^6 spleen cells were allowed to adhere in the tubes in which

Table 17

SUPPRESSION BY ADHERENT CELLS FROM TUMOUR-BEARING ANIMALS ON ^{14}C -THYMIDINE INCORPORATION OF T CELL-ENRICHED
SPLEEN CELLS INCUBATED WITH MTV

donors of test cells		T cell-enriched spleen cells		inhibition (%)	Significance
Mouse strain	Tumour weight (g)	adherent cells from the same animal cpm \pm SE*	normal adherent cells cpm \pm SE		
BALB/cfC3H	1.4	1,428 \pm 128	2,371 \pm 23	40	p < 0.001
	3.8	1,727 \pm 256	3,579 \pm 420	52	p = 0.025
GR	2.8	1,025 \pm 76	2,084 \pm 234	51	0.025 < p < 0.050
	4.2	1,810 \pm 190	3,534 \pm 337	49	0.005 < p < 0.010

* SE: standard error

the LS assay was performed; the supernatant was then removed and the adherent cells were washed three times with medium. The approximate number of adherent cells was estimated by subtracting the number of cells that did not adhere from the original concentration.

In both GR and BALB/cfC3H mice bearing large MTV-induced tumours (2 g or larger), it was constantly observed that the T cell response was markedly depressed if adherent spleen cells derived from the same animals were added, as compared to cultures to which adherent cells derived from normal animals were added (table 17). When different concentrations of adherent cells derived from mice bearing large tumours were added to T cell-enriched fractions derived from mice bearing very small tumours, ^{14}C -thymidine uptake in the presence of MTV decreased gradually. This was not the case when adherent cells from normal animals were added (fig. 12).

To determine whether the cellular hyporeactivity seen at a tumour weight exceeding 2 g (Chapter III) can be attributed to suppressor cells, individual BALB/cfC3H mice bearing tumours of varying sizes were tested for the presence of such cells. Spleen cells (0.5×10^6) derived from a tumour-bearing mouse were added to T cell-enriched fractions from such animals. Cultures to which normal spleen cells were added in the same concentration served as controls. Both sets of cultures were incubated with MTV and per cent inhibition of ^{14}C -thymidine incorporation was calculated. The results are shown in fig. 13. From the results, it is clear that suppressor cells are present in animals bearing small tumours: the first significant inhibition is seen at a tumour weight of 0.5 g. Greatest inhibition is seen at a tumour weight between 0.5 and 1.5 g; at about 4 g, considerable inhibition also occurs.

In animals bearing small tumours, removal of adherent cells lead to a considerable increase in MTV-induced proliferation. In animals bearing large tumours, however, spleen cell cultures containing suppressor cells sometimes

Table 18

INFLUENCE OF REMOVAL OF ADHERENT SUPPRESSOR CELLS ON ^{14}C -THYMIDINE INCORPORATION BY SPLEEN CELLS OF
BALB/cfc3H MICE BEARING TUMOURS OF VARYING SIZE

Tumour weight	Cell population	Incubation with RLV cpm \pm SE*	Incubation with MTV cpm \pm SE	Stimulation (%)	Significance
1.40	Spleen cells	3,464 \pm 127	4,272 \pm 106	24	0.010 $< p <$ 0.005
	T cell-enriched	2,650 \pm 664	6,747 \pm 380	155	0.005 $< p <$ 0.001
2.90	Spleen cells	5,611 \pm 342	3,856 \pm 98	-	0.010 $< p <$ 0.005
	T cell-enriched	2,446 \pm 368	2,216 \pm 929	0	NS**

* SE: standard error

**NS: not significant

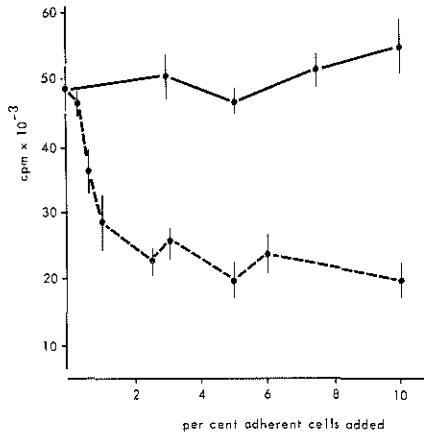


Figure 12:
Inhibition of cells responding to MTV by adherent suppressor cells in the LS assay

14. ³H-thymidine incorporation of T cell-enriched fractions derived from 3 BALB/cfC3H mice bearing small tumours (average 0.27 g), when varying numbers of adherent suppressor cells derived from 4 BALB/cfC3H mice bearing large tumours (average 3.10 g) were added. —: normal adherent cells added; ----: suppressor adherent cells added.

Vertical bars indicate SE of triplicate cultures. The RLV control was $6 \cdot 10^{-3}$ cpm. Shown in the figure are the results of one out of two experiments. The results of both experiments were similar.

gave significantly lower cpm values than did the RLV-control. Removing the suppressor cells by adherence to plastic surfaces gave about equal values for MTV and RLV cultures; even then, no MTV-specific proliferation was obtained. The average MTV-specific per cent stimulation of T cell-enriched fractions of 9 animals with small tumours (average 0.9 ± 0.2 g) was 83.1, and varied from 23 to 154; for 10 animals tested with large tumours (average 3.5 ± 0.2 g) this value was 15.2 with a variation of 0-62 %. Some representative results are shown on table 18.

Neither antigen nor antibody could be detected on the surface of adherent suppressor cells by membrane immunofluorescence studies.

Table 19

INFLUENCE OF EXTENSIVE WASHING AND TRYPSINIZATION ON MTV-SPECIFIC ^{14}C -
 THYMIDINE INCORPORATION OF INITIALLY UNRESPONSIVE T CELL-ENRICHED SPLEEN CELLS
 FROM TUMOUR BEARING BALB/cfC3H MICE*

Treatment of cells	T cell-enriched spleen cells		Percentage stimulation (+) or inhibition (-) \pm SE**
	Incubation with RLV cpm	Incubation with MTV cpm	
3 washings	2,587	1,884	- 25 \pm 8
8 washings	2,169	3,745	+ 73 \pm 23
Trypsinization	2,297	5,192	+126 \pm 24

* all values represent the mean of five identical experiments.

** SE: standard error of the mean values of five experiments

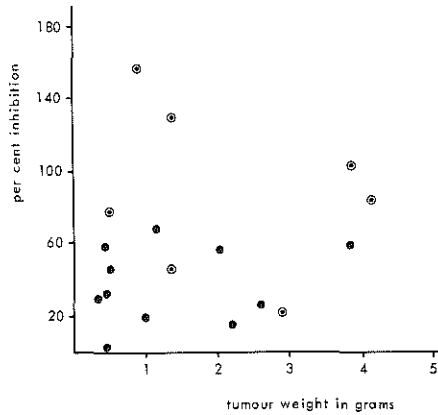


Figure 13:
Distribution of adherent suppressor cells in mice bearing tumours of varying size

The inhibition of ^{14}C -thymidine incorporation of T-cell enriched fractions from spleen cells of individual tumour-bearing BALB/cfC3H mice, caused by the addition of adherent suppressor cells derived from the same animal. Inhibition was expressed as percentage of identical cultures to which adherent cells derived from normal animals were added. ● : inhibition is not significant; ⊙ : inhibition is significant ($p < 0.025$).

5.2. Suppressive factor covering spleen cells

Since the fluctuations in the number of suppressor cells did not show a reverse correlation with the variation in the immune response during tumour growth, and since, in animals with very large tumours, removal of suppressor cells did not lead to recovery of specific anti-MTV proliferation, other inhibitory factors must be involved. It was investigated whether T cell unresponsiveness was due to coating of the cells with a suppressive agent. Therefore, T cell-enriched spleen cells were extensively washed or slightly trypsinized prior to testing.

T cell-enriched spleen cell fraction from tumour bearing BALB/cfC3H mice were unresponsive to MTV in the IS test after three washings. Significant proliferation occurred after mild trypsinization or after five additional

Table 20

EFFECT OF PREINCUBATION WITH WASH FLUID FROM UNRESPONSIVE SPLEEN CELLS ON THE
MTV-SPECIFIC ^{14}C -THYMIDINE INCORPORATION OF IMMUNE T CELL-ENRICHED SPLEEN
CELLS*

Preincubation with wash fluid from	Immune T cell-enriched spleen cells		Percentage stimulation (+) or inhibition (-) <u>+ SE**</u>
	Incubation with RLV cpm	Incubation with MTV cpm	
Normal spleen cells	2,052	4,777	+132 <u>±</u> 12
Spleen cells of tumour bearing BALB/cfC3H mice	2,537	4,974	+ 96 <u>±</u> 9
Spleen cells of tumour bearing BALB/c mice	3,088	1,552	- 50 <u>±</u> 7
Spleen cells of tumour bearing BALB/c mice	3,333	1,665	- 50 <u>±</u> 5

* all values represent the mean of four identical experiments.

** SE: standard errors of the mean value from four identical experiments

washings (Table 19). To confirm the hypothesis that washing removes a suppressive factor from the lymphocytes, the T cell fractions from animals with large tumours were washed eight times. T cell fractions (10^6 cells) from immunized animals were incubated with the wash fluid derived from 5×10^6 cells for 1 h at 37° C prior to testing. From the results in Table 20 it becomes evident that the wash fluid of the cells from tumour bearing animals inhibits MTV-specific proliferation, while the wash fluid derived from normal BALB/c spleen cells has no effect.

In all of the cases of BALB/cfC3H mice with tumours, trypsinization led to an augmentation of proliferation. The average increase was 84 %. Tumour weight varied between 0.16 and 4.20 g.

We also investigated the influence of the washing and trypsinization procedures on the indirect LAIF assay. LAIF production by unreactive spleen cells from tumours bearing BALB/cfC3H mice was not increased by trypsinization or repeated washings prior to testing. The significance of this finding remains, however, doubtful, since the reactivity of spleen cells from immunized animals was abolished after these treatments (table 21). It is possible that this inactivation is caused by removal of serum factors from the cells, which are required for LAIF-production. Therefore, LAI-tests were performed with trypsinized immune spleen cells after the addition of complement, and in the presence of 10 % normal mouse serum. However, these treatments did not lead to recovery of MTV-specific LAIF-production.

However, wash fluid of 100×10^6 spleen cells from tumour-bearing mice can inhibit the LAIF production by 20×10^6 spleen cells from immunized animals; the washings of normal cells had no effect on LAIF-production by immune spleen cells (Table 22). Probably, a similar suppressive agent as in the LS assay plays a role in the unresponsiveness of tumour-bearing animals in this test.

Table 21

INFLUENCE OF EXTENSIVE WASHING AND TRYPSINIZATION ON LAI REACTIVITY* OF SPLEEN CELLS
FROM TUMOUR BEARING AND IMMUNIZED BALB/c MICE

Donors	No. of experiments**	Treatment of cells	Spleen cell responses		
			Incubation with RLV no. of adherent cells	Incubation with MTV no. of adherent cells	Reduction of adherence (%) \pm SE***
Tumour bearing BALB/cfC3H mice	6	3 washings	92.1	83.3	9 \pm 2
		8 washings	67.9	66.2	2 \pm 2
		Trypsinization	57.9	56.8	2 \pm 2
Immunized BALB/c mice	4	3 washings	92.8	68.8	25 \pm 3
		8 washings	124.1	129.8	-
		Trypsinization	88.0	86.0	2 \pm 3

* Established by the indirect LAI-assay.

** Each figure represents the mean of the indicated number of identical experiments.

***SE: standard error of the mean value of the indicated number of identical experiments.

Table 22

EFFECT OF PREINCUBATION WITH WASH FLUID FROM UNRESPONSIVE SPLEEN
CELLS ON THE MTV-SPECIFIC LAI REACTIVITY* OF IMMUNE SPLEEN CELLS**

Preincubation with wash fluid from	Immune spleen cell responses		Reduction of adherence (%) \pm SE***
	Incubated with RLV (no. of adherent cells)	Incubated with MTV	
-	85.9	65.8	23 \pm 4
Normal spleen cells	243.5	199.9	18 \pm 2
Spleen cells of tumour bearing BALB/cfC3H mice	265.7	273.8	-
Spleen cells of tumour bearing BALB/c mice	105.8	113.4	-

* Established by the indirect LAI assay.

** Each figure represents the mean of four identical experiments.

*** SE: standard error of the mean value of four identical experiments.

Table 23

INFLUENCE OF SERUM FACTORS FROM TUMOUR BEARING BALB/cfC3H MICE ON LAI REACTIVITY* OF IMMUNE
SPLEEN CELLS

Tumour weight of serum donor (g)	Immune spleen cell responses		Significance	Quotient y^b
	normal serum + MTV (no. of adherent cells \pm SE ^a)	test serum + MTV		
1.1	75.0 \pm 4.0	104.5 \pm 3.9	p < 0.001	1.39
2.0	64.3 \pm 3.9	71.5 \pm 3.8	NS ^c	1.11
2.1	60.0 \pm 2.1	71.7 \pm 2.5	p < 0.010	1.20
3.0	82.3 \pm 2.3	124.5 \pm 3.3	p < 0.001	1.51
6.5	72.8 \pm 2.2	83.0 \pm 4.5	NS	1.14
6.8	54.8 \pm 1.6	64.1 \pm 1.5	p < 0.001	1.17

*Established by the indirect LAI assay

^aSE: standard error for 10 wells

^bquotient y: Chapter II,4

^cNS: not significant

5.3. Serum blocking factors

The presence of serum factors that block or enhance cellular immunity can be easily established with the indirect LAI assay. We tested 15 sera from tumour bearing BALB/cfC3H mice. In all tests, quotient y was higher than 1, which indicates blocking. The decrease in adherence when test serum was added was significant in about one-half of the cases. Blocking activity of the sera showed no relation to tumour weight (Table 23).

Sera that had blocking activity in the LAI assay also inhibited MTV-specific proliferation by immune T cell-enriched cell fractions. This is shown in Table 24.

5.4. Presence of MTV and anti-MTV antibodies in the suppressive factor and the blocking serum

The nature of the suppressive factors in wash fluids and sera from tumour-bearing mice was investigated with the Sepharose bead immunofluorescence assay. Prior to testing, the mouse sera were concentrated 10x by an Amicon-concentrator; the washings were derived from 200×10^6 cells per ml and concentrated 20x with the same technique. When the fluorescence of the lowest dilutions was about three times higher than background values, the samples were scored as positive (+); stronger fluorescence was recorded as ++, and when fluorescence was about equal to the positive control the samples were scored as +++. In both samples MTV could be detected (Table 25), although the levels were generally low. No MTV was detected in serum and wash fluid samples from normal animals. These results were confirmed by radioimmunoassay, kindly performed by Dr. A.A. Verstraeten (Netherlands Cancer Institute, Amsterdam).

By means of the Sepharose bead immunofluorescence assay anti-MTV antibodies were also detected in the spleen cell wash fluids from the tumour bearing mice, but were absent

Table 24

INFLUENCE OF SERUM FROM TUMOUR BEARING MICE ON MTV-SPECIFIC PROLIFERATION OF IMMUNE
T CELL-ENRICHED FRACTIONS

Serum donors	no. of experiments*	Immune T cell-enriched fraction responses		Percentage inhibition <u>±</u> SE**
		normal serum	test serum	
		+ MTV cpm	+ MTV cpm	
Tumour bearing BALB/cfC3H mice	4	3,834	2,738	-29 <u>±</u> 4
Tumour bearing BALB/c mice	2	3,620	2,442	-32 <u>±</u> 12

* Each figure represents the mean of the indicated number of identical experiments.

** SE: standard error of mean value of the indicated number of experiments

in that from normal BALB/c animals. The serum from tumour bearing BALB/cfC3H mice was positive for the anti-MTV antibodies, but the serum from tumour bearing BALB/c mice was negative, similar to sera from normal mice. Also the antibody levels found in the wash fluids and sera from tumour-bearing animals were rather low.

5.5. Discussion

In the present study, it was demonstrated that the MTV specific response in tumour bearing animals may be depressed by the presence of adherent cells. Increasing the cell density by adding an equal number of normal adherent cells had no inhibitory effect; therefore, it is not likely that the suppressive effect is due to a quantitative change in the spleen cell macrophage population of the tumour bearing mice (Kruisbeek and Van Hees, 1977). The suppressive effect was already seen when about 0.5 % adherent suppressive spleen cells were added to a cell population responding to MTV and about 5 % was enough to reach the maximal inhibitory effect. These results are in accord with those of Glaser et al. (1975) in rats bearing progressively growing Gross leukemia virus induced tumours.

Kirchner et al. (1974, 1975) observed a good correlation between the growth pattern of Moloney sarcoma virus-induced tumours and adherent suppressor cells; these cells were detected before the tumour had reached an appreciable size. In our investigation, suppressor cells could already be demonstrated at a tumour weight of 0.5 g. The greatest inhibitory effect was seen at a tumour weight between 0.5 and 1.5 g; there was also a considerable suppression at 4 g. The decline in spleen cell reactivity in the LAI and LS test when the tumour exceeds one gram in weight can be attributed to an increasing inhibition by suppressor cells. At 2 g, when the spleen cell activity in the LS test is at a minimum (Chapter III), the maximum inhibition by the

Table 25

PRESENCE OF MTV AND ANTI-MTV ANTIBODIES IN SUPPRESSIVE AGENTS AS
MEASURED BY THE SEPHAROSE BEAD IMMUNOFLOUORESCENCE METHOD*

Sample	Binding to anti-MTV antibodies	Binding to MTV
Serum from		
Normal BALB/c	-	-
Tumour bearing BALB/cfC3H	+	+
Tumour bearing BALB/c	++	-
Wash fluid of spleen cells from:		
Normal BALB/c	-	-
Tumour bearing BALB/cfC3H	+	+
Tumour bearing BALB/c	+	+

* When the fluorescence of the lowest dilutions was about three times higher than background values, the samples were scored as positive (+); stronger fluorescence was recorded as ++, and when fluorescence was about equal to the positive control the samples were scored as +++.

suppressor cells has already disappeared.

Removal of adherent cells did not result in a significant MTV-directed T cell response at heavy tumour weights in most cases. Probably, suppression by the adherent cells is gradually replaced by suppression by antigen-antibody complexes during tumour growth.

The mode of action of suppressor cells remains unclear. We could not detect antigen or antibody on the surface of the suppressor cells by membrane immunofluorescence studies. Eggers and Wunderlich (1975) found no evidence for the mediation of suppression by soluble factors.

We did not investigate whether the suppressor cells in the MTV-system are adherent T cells. Until now only aspecifically suppressive T cells with adherent properties have been recognized (Waksman, 1977). Suppressor cells are generally found in cases in which animals are overloaded with antigen and the possibility must be considered that they are part of a tolerance inducing mechanism that is activated when the immune system is confronted with excess tumour antigen.

It was demonstrated that MTV-specific proliferation is greatly increased when T cell-enriched cell fractions from tumour bearing mice are extensively washed; slight trypsinization of the cell surface resulted in even higher responses. Medium in which the unresponsive spleen cells from tumour bearing donors were washed repeatedly contained factors which suppressed the MTV-specific proliferation of immune T cell-enriched fractions.

In our system trypsinization and repeated washings completely abolished the LAI reactivity of immune spleen cells. This deficit could not be restored by adding complement or by performing the test in 10 % normal mouse serum; possibly a receptor necessary for the reaction, in which cell communication is mediated by soluble factors (Chapter IV), is removed or destroyed by these treatments. However, the washings of spleen cells from tumour bearers inhibited

the LAI reactivity of untreated spleen cells from immunized donors. This indicates that LAI reactivity can be blocked by suppressive factors attached to the cell surface of immunocytes from tumour-bearing animals.

Factors that block cellular reactivity are also present in the serum of tumour bearing mice; these factors again inhibited both the MTV-specific LAI reactivity and proliferation.

With the Sepharose bead immunofluorescence assay it was established that the blocking serum as well as the wash fluids from spleen cells of tumour bearers contained MTV, whereas serum and wash fluid derived from normal animals did not. The wash fluid and one of the blocking sera of tumour bearers also contained anti-MTV antibodies. Most likely, both suppressive mechanisms are due to antigen-antibody complexes. The levels of MTV antigens and anti-MTV antibodies were very low, but this is likely to be due to the immune complexes interfering with the amount of antigen and antibody measured in this system (Matthews et al., 1975).

Many authors have suggested that blocking factors in sera of individuals with cancer are the result of free antigen or immune complexes. Gorczynski et al. (1975) presented evidence that both free antigen and antigen-antibody complexes mediate tumour-specific blocking, whereas nonspecific blocking is mediated by only antigen-antibody complexes. They also suggested that the suppressive action of adherent cells is mediated by the release of antigen-antibody complexes in culture.

At the moment there is no insight into the role of immune complexes in immunodepression. There is a growing body of evidence that in immune response mechanisms cell-to-cell interactions are often mediated by soluble factors; to exert their influence, all of these factors need to interact with the cell membrane. It is tempting to specula-

te that the suppressive effects are due to an excess of antigen-antibody complexes: Antibody and antigen, whether or not complexed, will bind to the Fc and antigen receptors on lymphoid cells. When many immune complexes are in the circulation, these may subsequently attach to the antigen, antibody, or complexes bound on the cell membrane. When this process continues, at the end the cell will become covered with a reticulated formation which mask lymphokine acceptor sites.

The question as to why macrophages do not phagocytose these immune complexes (McKeever et al., 1976) is of crucial importance from both a practical and a theoretical point of view.

CHAPTER VI

MTV-PROTEIN VACCINE: INDUCTION OF ANTIVIRUS IMMUNITY AND ENHANCEMENT OR INHIBITION OF TUMOUR-SPECIFIC TRANSPLANTATION RESISTANCE

Vaccination with oncoviral subunit proteins may protect mice against tumour development. In the MTV system several vaccination studies with tumour antigens have been performed. However, these studies yielded conflicting results: some experiments resulted in protection against mammary tumour development, whereas other trials led to accelerated tumour development (Chapter I;5). We were interested in the question whether successful vaccination can be achieved by treatment with the major membrane glycoprotein of MTV, gp52, which is also expressed on the mammary tumour cell membrane. At the time these studies were started, there were no known methods to purify gp52; therefore, these vaccination experiments were performed with an MTV-protein fraction approximately 60 % of which consisted of gp52 (Chapter II;8). This vaccine was derived from MTV-S. Several doses of this protein fraction were employed.

Studying the effect of a given vaccination procedure on spontaneous tumour development requires long-term experiments, and is complicated by the need to force breed the mice. Therefore, in this exploratory study we used the take of transplanted tumours as a measure for prophylaxis against spontaneous tumour development. Most of these investigations were performed with the DBA/1 leukaemia L1210 which grows in ascites form and carries MTV antigens (Radzikowski et al., 1972). The advantage of this cell line is the rapidity of tumour testing and the quantitative aspects of the assay. A few days' delay in animal kill can be expressed in terms of per cent kill of the inoculated tumour cells (Skipper et al., 1964). However, the L1210

might represent a very artificial system, with little relevance to the control of spontaneous mammary carcinogenesis. We therefore also studied the induction of transplantation resistance in BALB/c mice to an MTV-O induced mammary tumour which has the disadvantage of a long observation period. We used alum and IPM as adjuvants; controls were treated with the adjuvant alone.

Since the vaccination procedures might induce immune suppressive mechanisms, it was important to establish the immunologic events after the vaccination had been performed. The immune response was therefore monitored on days 12 and 20 after vaccination. MTV-specific cellular immune reactivity and the presence of serum blocking or enhancing factors was established by means of the LAI assay. The presence of anti-MTV-antibodies was established by means of membrane immunofluorescence. At the time of vaccination, the mice were 10-12 weeks of age. All vaccines were injected intraperitoneally. The appearance of the L1210 leukaemia and mammary tumours in the mice was established as described in Chapter II;8.

6.1. Latency period of tumours after injection and influence of adjuvants

For the estimation of the effects of adjuvants and vaccine on tumour cell kill the latency period between injection and appearance of the two neoplasms first had to be determined. The presence of tumours was established as described in Chapter II;8. Fig. 14a presents the average latency period (in weeks) of MTV-O induced mammary tumours in the BALB/c strain; the latency period is plotted against the number of tumour cells in the inoculum. The latency period was linearly related to the logarithm of the cell inoculum. When 10^6 cells were grafted, tumours appeared within 4 weeks. All of the animals produced tumours when 10^4 cells were inoculated, but the average latency time was 7 weeks. When 10^3 cells were injected, 33 % of the

animals developed tumours within the observation period of 30 weeks.

In our experiments, the L1210 leukaemia showed a rapid killing of the grafted animals (fig. 14b). One log difference in cell number caused a difference of 1 - 1.5 days in the killing of the animal. At low cell numbers (below 100) many survivors were noted up to 5 weeks; with 50 cells 60 %, and with 25 cells 80 % of the animals survived. The latent period in the animals that developed tumours was then considerably lengthened. This deviation from usual titration patterns of L1210 (Skipper et al., 1964) was probably due to the *in vitro* cultivation of the line (P. Lelieveld, personal communication).

The effect of 50 µg IPM on the latency period after inoculation with 10^4 , 10^3 or 10^2 L1210 cells corresponded with approximately a 1-day delay. There were no survivors in any group. This indicates that not a given number, but a given proportion of the inoculated L1210 cells is killed by the activated immune system. For this reason it seemed reasonable to express the protective effect of a given vaccination procedure in percentage kill of the inoculated tumour cells. When accelerated growth of the tumour occurred, we expressed this in percentage "gain" of the inoculated tumour cells (i.e. less tumour cells were killed by the immune system in vaccinated animals than in the controls).

The effect of injection with alum only is presented in Table 26. For both the mammary tumour and the leukaemia, the latency period seemed to be considerably lengthened. On the basis of fig. 14, the per cent kill of inoculated tumour cells has been extrapolated. For the leukaemia this was approximately 40 % at different doses of alum; only when a dose of 100 µg was employed less protection was observed. A 90% kill of inoculated tumour cells was found for the mammary tumour given 5 µg alum, but at higher doses of this adjuvant the kill rate was decreased. Since the adjuvants influenced tumour growth, in all experiments

Table 26

EFFECT OF INJECTION OF DIFFERENT DOSES OF ALUM ON GROWTH OF TUMOUR CELLS TRANSPLANTED 20 DAYS AFTER INOCULATION

Strain	Tumour	Dose of alum μg	No. of inoculated cells	Survival		Average latency period ^a		Calculated cell kill, %
				T ^b	U ^c	T	U	
BALB/c	mammary tumour	5	10 ⁴	0/7	0/9	9.7 \pm 0.8 wk ^d	7.0 \pm 0.2 wk	90
		50	10 ³	5/7	3/9			50
			10 ⁴	3/10	0/9		7.0 \pm 0.2 wk	90
		500	10 ³	2/5	3/9			10
DBAF	L1210 leukaemia	0.5	100	2/5	0/10		13.2 \pm 1.0 days	45
		5	250	0/7	0/10	13.6 \pm 0.3 d ^d	12.8 \pm 0.0 days	64
			200	0/5	0/10	14.3 \pm 0.0 d	12.9 \pm 0.0 days	66
			100	0/5	0/10	14.2 \pm 0.3 d	13.2 \pm 1.0 days	30
		50	250	0/13	0/10	13.2 \pm 0.1 d	12.8 \pm 0.0 days	40
		100	50	5/7	8/14			17

^a Values are means \pm SE. The presence of tumour was established as described in Chapter II.8

^b T = treated animals

^c U = untreated animals; figures derived from figure 14.

^d wk = weeks; d = days.

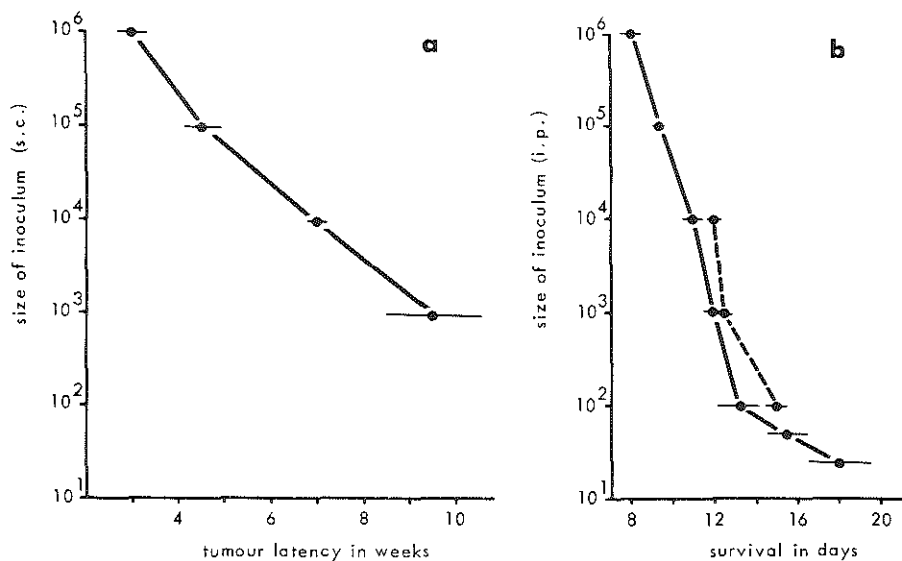


Figure 14:

Latency periods until time of tumour appearance after inoculation with various numbers of tumour cells

Horizontal bars indicate standard error of the average time of tumour appearance (mammary tumour) or mortality of mice (L1210) as calculated from 9 (A) or 10 (B) animals. Establishment of tumour appearance: see Chapter II; 8.

- A: Average latency of tumour appearance in 12-week-old female BALB/c mice after sc inoculation with tumour cells derived from an MTV-O induced tumour.
- B: Average survival time of 12-week-old female DBA/f mice after ip inoculation with L1210 cells grown at least 2 days *in vitro*.
 —: without treatment of mice; ---- mice given ip injections of 50 ug IPM 20 days before inoculation with tumour cells.

described below the controls were treated with the adjuvant.

6.2. Effect of vaccination on the growth of transplanted mammary tumours in BALB/c mice

The results are summarized in Table 27. The challenge dose was varied to avoid too much survival in the control groups, in which the varying doses of alum had a protective

Table 27

EFFECT OF INJECTION OF DIFFERENT DOSES OF A GP52-ENRICHED MTV PROTEIN FRACTION ON GROWTH OF TRANSPLANTED MAMMARY TUMOUR CELLS IN BALB/c MICE

Group no.	Treatment	No. of inoculated cells	Survival		Average latency period in weeks ^a			Calculated cell kill (-) or gain (+), %
			v ^b	c ^c	v	c	Significance	
1	1 µg MTV protein and 5 µg alum	10 ⁴	0/7	0/7	20.3 ± 2.6	9.7 ± 0.8	0.001 < p < 0.005	-99.9
2	10 µg MTV protein and 50 µg alum	10 ³	4/7	5/7				+30
		10 ⁴	4/7	2/5				-42
3	10 µg MTV protein and 50 µg alum; 2 boosts of 1 µg MTV protein	10 ⁴	3/7	1/5				-50
4	100 µg MTV protein and 500 µg alum	10 ³	0/5	2/5	11.1 ± 0.3	16.2 ± 0.4	p < 0.001	+40

^a Values are means ± SE of weeks of tumour appearance; tumours were established as described in Chapter II.8

^b v = animals treated with MTV protein and adjuvant

^c c = control animals, treated with adjuvant alone

effect. As compared to the control given alum alone, the vaccination with 1 µg MTV protein fraction enriched for gp52 led to a significant delay in the onset of the transplanted tumours (20 vs. 10 wk). This corresponded with a 3-log kill (99.9 %) of the inoculated tumour cells. When 10 µg of this vaccine was injected, no clear effect was observed. An additional booster dose of 1 µg gp52 on days 7 and 14 had no significant effect. When 100 µg of the vaccine was used, tumours appeared considerably earlier in the vaccinated animals than in the controls. No influence on tumour growth was observed when BALB/c mice, given injections of 1 and 100 µg gp52-enriched protein precipitated on alum, were challenged sc with 10^4 MOPC-315 plasmacytoma cells (average latency period with 1 µg: 19.7 days; controls, 19.5 days; with 100 µg: 19.7 days; controls, 19.6 days).

6.3. Effect of vaccination on mortality of L1210 leukaemia in DBA/ mice

Table 28 summarizes the results obtained with alum as an adjuvant. Again, the challenge dose was varied to avoid too much survival in the control groups due to the protective action of the adjuvants. When complete virus, disrupted by freezing and thawing ten times, was used as antigen, doses of both 1 and 10 µg tended to induce a slight protection.

When the gp52-enriched protein fraction was used, however, tumour growth was already accelerated at very low doses (0.1 and 1 µg). The high dose of 80 µg also enhanced tumour growth; only 10 µg was protective.

The results obtained with IPM as an adjuvant are outlined in Table 29. When 50 µg IPM was used, a protective effect, which diminished with an increasing dose of virus protein, could be observed for the MTV protein vaccine. However, when different doses of gp52 were injected with 100 µg IPM, death was accelerated.

Table 28

EFFECT OF INJECTION OF DIFFERENT DOSES OF ANTIGEN PRECIPITATED ON ALUM IN DBA/1 MICE ON GROWTH OF L1210 LEUKAEMIA

Group no.	Treatment	no. of inoculated cells	Survival		Average days of survival ^a			Calculated cell kill (-) of gain (+), %
			v ^b	c ^c	v	c	Significance	
1	1 µg disrupted virus and 5 µg alum	250	1/5	0/7	14.0 ± 0.1	13.6 ± 0.3	NS ^d	-20
2	10 µg disrupted virus and 50 µg alum	250	0/5	0/13	14.0 ± 0.3	13.2 ± 0.1	p < 0.010	-15
3	0.1 µg MTV-protein and 0.5 µg alum	100	1/10	2/5				+50
4	1 µg MTV-protein and 5 µg alum	100	0/10	0/5	13.1 ± 0.2	14.2 ± 0.3	p < 0.010	+30
5	1 µg MTV-protein and 5 µg alum; 1 boost 1 µg MTV-protein	200	0/9	0/5	14.0 ± 0.0	14.3 ± 0.0	p < 0.001	+10
6	10 µg MTV-protein and 50 µg alum	250	0/8	0/13	14.0 ± 0.3	13.2 ± 0.1	p < 0.001	-15
7	80 µg MTV protein and 100 µg alum	50	1/8	5/7				+80

^a Values are means ± SE; death was taken as a measure for tumour development.

^b v = animals treated with antigen and adjuvant

^c c = control animals treated with adjuvant alone

^d NS = not significant

Table 29

EFFECT OF INJECTION OF DIFFERENT DOSES OF A GP52-ENRICHED MTV PROTEIN FRACTION AND IPM IN DBA/2 MICE ON GROWTH OF L1210 LEUKAEMIA

Group no.	Treatment	No. of inoculated cells	Survival		Average days of survival ^a			Extrapolated cell kill (-) or gain (+), %
			v ^b	c ^c	v	C	Significance	
1	0.1 µg MTV protein and 50 µg IPM	100	2/10	0/5	16.9 ± 0.6	14.9 ± 0.2	p = 0.025	-20
2	1 µg MTV protein and 50 µg IPM	100	0/10	0/5	16.3 ± 0.5	14.9 ± 0.2	NS ^d	-10
3	80 µg MTV protein and 50 µg IPM	50	6/10	4/7				- 5
4	1 µg MTV protein and 100 µg IPM	200	0/9	1/6	14.9 ± 0.5	15.9 ± 0.3	NS	+10
5	10 µg MTV protein and 100 µg IPM	200	0/9	1/6	13.4 ± 0.2	15.9 ± 0.3	p < 0.001	+25

^a Values are means ± SE; death was taken as a measure for tumour appearance.

^b v = animals treated with antigen and adjuvant

^c C = control animals treated with adjuvant alone

^d NS = not significant

Table 30

IMMUNOLOGIC REACTIVITY AFTER TREATMENT WITH VACCINES BEFORE AND AFTER SUBSEQUENT CHALLENGE WITH TUMOUR CELLS IN
BALB/c MICE^a

Group	Vaccines	Day after vaccination	LAI % reduction ^b	Blocking-enhancing serum factors, quotient y^b	Membrane fluorescence positive serum dilution	Calculated cell kill (-) or gain (+), %
1	1 μ g MTV protein and 5 μ g alum	22 ^c	7	0.98	16	-99.9**
2	10 μ g MTV protein and 50 μ g alum	16	13*	0.92	8	-12
		25 ^c	20*	1.22*	16	
3	10 μ g MTV protein and 50 μ g alum + 2 boosts 1 μ g MTV protein	20	21*	0.90*	2	-50
		25 ^c	2	1.09	2	
4	100 μ g MTV protein and 500 μ g alum	16	19*	1.02	4	+40**
		25 ^c	5	1.19*	16	

^a Controls were negative in all tests before challenge. Values after challenge: see text, page 107

The controls were treated with alum only.

^b See Chapter II.3.4

^c After challenge

* Reactivity is significant ($p < 0.010$)

** Difference in latency period between the vaccinated and the control group was significant (Table 27).

6.4. MTV-specific immunologic reactivity of vaccinated animals

The immunologic reactivity to MTV in vaccinated BALB/c mice is summarized in Table 30. Cellular immune reactivity and the presence of serum factors that interfere with cellular reactivity were estimated with the direct and the indirect LAI-assay, respectively. MTV-S was used as the antigen; RLV served as a specificity control. Anti-MTV antibodies were estimated by membrane immunofluorescence on MTV-positive AR 963 cells; RLV-infected NIH 3T3 cells served as a specificity control.

BALB/c mice vaccinated with 1 μ g MTV protein plus alum had a weak positive LAI after challenge with the tumour but lacked blocking factors in their sera. Tumour growth was considerably delayed in this group.

In group 2 (10 μ g MTV-protein plus alum) after challenge, the LAI reaction was positive, but blocking factors were also present; the controls also showed significant blocking (quotient y was 1.26). No significant protection against the tumour was noticed in this group.

In group 3, which received booster doses of virus protein, the LAI-reaction was negative after challenge with the tumour, but, in contrast to the control group, no blocking factors were found in the sera. A slight increase in survival was observed in this group.

In the last group, which received a high dose of vaccine (100 μ g), the LAI-reaction became negative after challenge with tumour cells and blocking factors appeared in the sera. Tumour growth was significantly accelerated in this group.

Before grafting, the control animals of all groups had no antibodies in their sera and they did not show LAI reactivity or influence of serum factors on cellular immunology.

Table 31

IMMUNOLOGIC REACTIVITY IN DBA/1 MICE AFTER TREATMENT WITH ANTIGEN AND ALUM^a

Group no.	Vaccines	Day after vaccination	LAI % reduction ^b	Blocking-enhancing serum factors, quotient y ^b	Membrane fluorescence, positive serum dilution	Calculated cell kill (-) or gain (+), %
1	1 µg disrupted virus and 5 µg alum	15	9*	1.05	8	-20
		20	17*	1.03	16	
2	10 µg disrupted virus and 50 µg alum	15	14*	0.67*	16	-15**
		20	23*	0.96	16	
3	0.1 µg MTV protein and 0.5 µg alum	13	15*	0.94	16	+50
		20	15*	1.36*		
4	1 µg MTV protein and 5 µg alum	13	8	1.09	8	+30**
		20	18*	1.20*	16	
5	1 µg MTV protein and 5 µg alum; 1 boost of 1 µg MTV protein	17	13*	1.00	8	+10**
		26 ^c	12*	1.19	4	
6	10 µg MTV protein and 50 µg alum	15	17*	0.62*	16	-15**
		20	26*	2.00*	32	
7	80 µg MTV protein and 100 µg alum	12	5	1.28*	32	+80

^a Controls, treated with alum only, were negative before challenge in all tests (page 109).

^b See Chapter II, 3, 4

^c After challenge

* Reactivity is significant ($p < 0.010$)

** Difference in survival time between vaccinated and control groups is significant (Table 28).

The antiviral immunologic reactivity of DBAf mice treated with antigen in combination with alum is presented in Table 31. When virus disrupted by freezing and thawing ten times was used as an antigen (groups 1, 2), LAI reactivity was significant. Serum blocking factors were absent. After inoculation of a dose of 10 μ g disrupted MTV, enhancing factors could be demonstrated in the serum. Both groups seemed to display a slight protection against tumour growth (Table 28).

When the MTV protein fraction in combination with alum was used, LAI reactivity was absent only with the highest dose (80 μ g). However, blocking factors could be demonstrated in all groups. Positive antibody titers became higher with increasing vaccine dose. Except for a dose of 10 μ g, all groups showed accelerated tumour growth.

Table 32 shows the data on immunologic reactivity of DBAf mice treated with different doses of the MTV protein fraction and IPM. All groups, except those receiving 80 μ g of the protein fraction, showed good cellular immunologic reactivity. Serum blocking factors were present in all groups. Groups 1 and 2, which showed significant protection, and groups 4 and 5, which showed accelerated tumour growth, had low antibody titers.

DBAf control mice treated with alum or IPM only were negative before challenge in either immunologic test, but thereafter became positive in the LAI assay. Their sera then showed blocking activity.

6.5. Discussion

The procedure for isolation of MTV virions from tumours, yields electron microscopically clean virus preparations. The polypeptides of B-type particles isolated by this procedure are, after identification by PAGE, similar to that of tissue culture-derived virus (Teramoto et al., 1974). However, host proteins seem to contaminate the virus

Table 32

IMMUNOLOGIC REACTIVITY IN DBAF MICE AFTER TREATMENT WITH DIFFERENT DOSES OF MTV PROTEIN FRACTION AND IPM^a

Group no.	Vaccines	Day after vaccination	LAI, % reduction ^b	Blocking-enhancing serum factors, quotient y ^b	Membrane fluorescence, positive serum dilution	Calculated cell kill (-) or gain (+), %
1	0.1 µg MTV protein and 50 µg IPM	13	15*	1.61*	16	-20**
		20	9	1.58*	64	
2	1 µg MTV protein and 50 µg IPM	13	13*	1.09	32	-10
		20	10*	1.23*	128	
3	80 µg MTV protein and 50 µg IPM	12	6	1.12*	4	- 5
4	1 µg MTV protein and 100 µg IPM	17	18*	1.20*	1	+10
		26 ^c	24*	1.15	2	
5	10 µg MTV protein and 100 µg IPM	17	21*	1.26*	0	+25**
		26 ^c	28*	1.08	16	

^a Controls, treated with alum only, were negative in all tests before challenge. See text, page 109.

^b See Chapter II.3.4

^c After challenge

* Reactivity is significant ($p < 0.010$)

** Difference in survival time between vaccinated and control group is significant (Table 29).

preparations: immunoelectron microscopy revealed that normal cell surface antigens are present in the virus envelope (Calafat et al., 1976). In addition, host proteins might be sticking to the virions. After absorption, little or no host proteins are assumed to be present in the preparation. Unfortunately, as became evident later, the vaccine is contaminated with Con A subunits. Such contamination can be prevented by extensive prewashing of the Con A Sepharose columns. Preliminary results of studies with purified MTV proteins indicate that the Con A subunits have no effect on the resistance to transplanted tumours and on immunologic reactivity to MTV.

In this exploratory study, each variable such as cell number, protein dose, and adjuvant dose was not studied exhaustively; a consistent pattern emerges, however, which provides a basis for further detailed study: A clear dose effect exists for the MTV protein fraction. Protection is found only with low doses of MTV protein. The protective effect diminishes with increasing dose. High doses may result in enhanced tumour growth.

Dose and kind of adjuvant are important. A high dose of IPM, which is protective by itself, accelerates tumour growth when combined with a dose of vaccine that in other circumstances would delay tumour growth. Obviously, when a strong adjuvant is used, little antigen is needed to tip the balance in favour of tumour acceleration.

Results obtained with one system (MTV-O induced mammary tumours in BALB/c mice) are not directly comparable to those obtained with another (L1210 leukaemia in DBA/1 mice). For example, in the first instance a low dose of vaccine induced clear protection, whereas in the other enhanced tumour growth was observed. This situation might be due to differences in the systems (sc solid tumour vs. ascites leukaemia). Also strain differences will play a role in immunologic reactivity: before challenge with the L1210 leukaemia, vaccinated DBA/1 mice always developed blocking factors, whereas vaccinated BALB/c mice were free of them.

In our study, vaccination with virus disrupted by freezing and thawing induced protection far more efficiently than did the fraction enriched for gp52, in which all proteins were solubilized. Charney et al. (1976) and Stutman (1976) obtained good results with a formalinized MTV preparation. The latter, however, observed tumour enhancement after vaccination with soluble antigens extracted from a C3H mammary tumour. We are presently investigating whether structural integrity of the virus envelope contributes to protection against tumour growth.

Protection is anticipated to result from the interaction of cellular immunity and humoral factors, i.e., blocking or enhancing factors and antibodies.

The specificity of the LAI-assay is discussed in Chapter II.3. Specificity of the antibodies, as detected by the membrane immunofluorescence test, was deduced from the negative reactions of normal mouse sera with the cell line AR 963 and the use of RLV-infected cells as a control, inasmuch as the MTV-producing cell line also shows some C-type particles (Van Pelt et al., 1976).

When the LAI was positive and blocking factors were absent, protection against tumour growth was good. The combination of low cellular reactivity and serum blocking factors has the adverse effect of faster tumour growth than in control animals. Predictability is more difficult for the two other combinations: positive LAI plus blocking factors or negative LAI and no blocking factors. In this case, antibody titer will probably play a crucial role, as evident in the experiments in which IPM was used as an adjuvant (Table 32): the groups with high antibody titers showed protection against tumour growth despite significant amounts of blocking factors. Summarizing, our results suggest the following relation between the immune response on day 20 after vaccination, and protection against or enhancement of the growth of a later tumour challenge:

<u>LAI-reactivity</u>	<u>serum blocking factors</u>	<u>anti-MTV antibodies</u>	<u>effect on tumour growth</u>
<u>+</u>	-	<u>+</u>	} protection
+	+	+++	
+	+	-	} no clear effect
-	+	+	
+	++	-	} enhancement
-	+	-	

The tumour growth acceleration induced by high doses of MTV protein is obviously due to the earlier appearance of blocking factors and poor cellular reactivity. That blocking factors are lacking when virus is used as an antigen is also of interest.

This exploratory study shows that protection against transplanted tumours by an MTV-protein vaccine occurs only when the induction of blocking factors by the vaccination procedure is prevented. This is more likely to be achieved when a low dose of vaccine is used. Therefore, low doses of MTV subunit vaccines might be useful for the establishment of prophylaxis or at least delay of primary tumour development in mice.

CHAPTER VII

MTV PROTEIN VACCINE: INFLUENCE ON PRIMARY MAMMARY TUMOUR DEVELOPMENT

The results from the vaccination studies on the induction of resistance against transplanted tumour cells (Chapter VI) indicate that a low dose of vaccine only may yield protection against primary mammary tumour development. For this reason, we tested the effect of a low dose (10 µg) without adjuvant on primary mammary tumour development in female mice. The low MTV expressing C3Hf and BALB/c, and the high MTV expressing GR and BALB/cfC3H strains, were employed. We used the gp52-enriched MTV-S vaccine, that was also tested in Chapter VI; the preparation of this vaccine is described in Chapter II;8.

At the time of vaccination, the animals were 8 weeks of age; at the age of 22 weeks, they were subjected to forced breeding, since the resulting hormonal stimulation promotes tumour development. Tumours were established as described in Chapter II;8.

Cellular reactivity to MTV was established with the direct LAI assay; reactivity to tumour cells was demonstrated with the Winn test (Winn, 1961). Anti-MTV-antibodies were detected by means of membrane immunofluorescence. These vaccination studies are very long lasting and were started 3 years ago. At that time, the role of blocking factors in MTV directed cellular immunity was not yet known to us. Therefore, the induction of blocking factors by the vaccination procedure has unfortunately not been established.

The vaccination studies reported in chapter VI yielded no clear results concerning the effect of boosting on the induction of resistance to transplanted mammary tumours.

Table 33

INFLUENCE OF VACCINATION WITH 10 µg MTV-S PROTEIN VACCINE ON DEVELOPMENT OF PRIMARY MAMMARY
TUMOURS IN DIFFERENT MOUSE STRAINS^a

Mouse strain	Tumour incidence		Latent period (weeks) \pm SE ^b		
	Vaccinated	Controls	Vaccinated	Controls	Significance
GR	9/ 9 (100 %)	54/54 (100 %)	36.0 \pm 2.7	27.1 \pm 1.2	0.005 < p < 0.010
C3Hf	15/39 (39 %)	28/90 (31 %)	57.7 \pm 4.2	76.9 \pm 2.5	p < 0.001
BALB/c	39/107(37 %)	27/90 (30 %)	66.9 \pm 3.2	77.4 \pm 3.1	0.010 < p < 0.025
BALB/cfC3H	29/31 (94 %)	38/40 (95 %)	42.0 \pm 1.6	36.4 \pm 0.9	0.001 < p < 0.005

^a: design of experiments: see Chapter II.6

^b: standard error

Therefore, it was investigated whether the administration of booster doses have a beneficial effect on primary tumour development. In these experiments, 16-week-old BALB/cfC3H females were vaccinated with an MTV-O derived protein fraction obtained by the same purification procedure (Chapter II.8), that also contained approximately 60 % gp52.

For the initial immunization we used a dose of 10 µg that was precipitated on alum. Subsequently, 0, 2 or 5 booster doses of 1 µg unprecipitated MTV-O protein fraction were injected. The animals were subjected to forced breeding when they were 37 weeks of age.

Since limited numbers of mice were available, the sera were tested only for the presence of anti-MTV antibodies.

7.1. Influence of an MTV-S protein vaccine on tumour incidence and immunity in female BALB/c, BALB/cfC3H, C3Hf and GR mice

Effect of vaccination on tumour incidence. A single sc immunization with 10 µg protein vaccine resulted in a significant delay in tumour appearance in the high cancer strains GR and BALB/cfC3H. It is important to note that the incidence of tumours was not decreased. In the C3Hf and the BALB/c strain, which normally have a moderate incidence of tumours appearing at a relatively old age, vaccination resulted in accelerated tumour development and a slight increase in tumour incidence. In the C3Hf strain the shortening of the latent period for tumour development is highly significant; in the BALB/c strain the rather low P-value represents the border line of significance (table 33).

For the interpretation of vaccination studies it is important to know whether transplantation resistance to syngeneic mammary tumours is a useful parameter. Therefore, GR and BALB/c mice vaccinated with 10 µg of the MTV-S

Table 34

IMMUNOLOGIC REACTIVITY OF ANIMALS VACCINATED WITH 10 µg MTV-S PROTEIN FRACTION^a

Mouse strain	weeks after vaccination	MTV-S specific LAI-reactivity (% reduction) ^b	mammary tumour cell specific membrane immunofluorescence (positive serum dilution)
GR	1	12*	1/ 2
	2	23*	1/32
	3	18*	1/ 8
	9	ND**	1/16
BALB/c	1	10*	1/ 2
	2	5	1/ 8
	3	7	1/ 8
	7	ND	1/ 2

^a: Each result is obtained with pooled peritoneal cells and pooled sera from two mice.

^b: see Chapter II,3

* reactivity is statistically significant ($p < 0.010$)

** ND: not done

protein vaccine and untreated control animals were inoculated with 10^4 syngeneic mammary tumour cells at twenty days post-vaccination. Both control and vaccinated animals all developed tumours within 4.6 to 5.4 weeks; although the number of mice in every group was rather large (17-32) no significant difference in latent period between treated and untreated animals could be detected.

Effect of vaccination on the immune response. At various intervals after treatment the vaccinated GR and BALB/c mice were tested for MTV-S specific cellular immune reactivity by means of the direct LAI-assay; the presence of mammary tumour cell-specific antibodies was established by means of membrane immunofluorescence on the A963 cell line. The tests were performed with pooled cells and sera derived from two mice. The results are summarized in table 34.

Anti-mammary-tumour cell antibodies as detected with the membrane immunofluorescence test appeared in somewhat higher titer in the GR than in the BALB/c strain. Untreated GR or BALB/c mice of the same age had no detectable antibodies in their serum. Cellular reactivity as estimated by the LAI-assay was clearly stronger in the GR than in the BALB/c strain. No appreciable reactivity was found in the latter strain two weeks after vaccination. For vaccination studies it is important to know whether anti-MTV cellular immune reactivity as measured by *in vitro* assays is a measure of the induced *in vivo* anti-tumour cell reactivity. We therefore also performed the Winn-test (Winn, 1961), in which spleen cells are tested for their ability to suppress the growth of admixed tumour cells in syngeneic recipients.

In the GR strain, both 25 and 5×10^6 spleen cells taken 40 days after immunization completely prevented the growth of 5×10^3 admixed MTV-P induced tumour cells. Normal GR spleen cells had no inhibitory effect on the growth of transplanted mammary tumours; tumour incidence

was 100 %. The average latency period was 4.5 weeks in the case of 5,000 normal spleen cells to 1 tumour cell and 5.0 weeks in the case of 1000 normal spleen cells to 1 tumour cell. Each group consisted of 10 mice; the observation period was 5 months.

Spleen cells from vaccinated BALB/c mice, however, only partially inhibited the growth of a tumour induced by MTV-O (fig. 15a; b). As to be expected, the inhibitory effect was

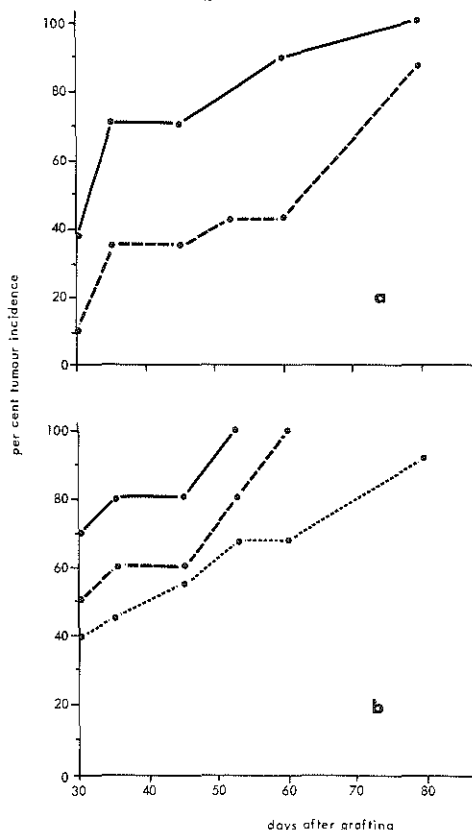


Figure 15:
Results of the Winn-assay with spleen cells from vaccinated BALB/c mice

Spleen cells were taken forty days after vaccination with 10 μ g MTV-S protein fraction, and mixed with MTV-O induced tumour cells at different ratios. Then the mixtures were injected sc into syngeneic recipients. The figure represents the tumour incidence in the recipients: A. The spleen cell : tumour cell ratio was 5000 : 1. -----: spleen cells from vaccinated mice; ———: spleen cells from normal mice. B. The lymphoid cell : tumour cell ratio was 1000 : 1.: T-cell-enriched spleen cells from vaccinated mice; -----: spleen cells from vaccinated mice.

more pronounced when the greatest number of spleen cells was given. When a T-cell-enriched spleen cell fraction was mixed with the tumour cells, the inhibitory effect was slightly increased, which might indicate that T cells play a crucial role in the mammary tumour cell directed cellular immune reactivity (fig. 15b).

7.2. Influence of different vaccination schemes on primary tumour development and antibody level in the BALB/cfC3H strain

Female BALB/cfC3H mice, which are naturally chronically infected with MTV-S, were vaccinated with the MTV-O protein fraction. For the primary immunization 10 µg of the protein fraction precipitated on alum was administered sc. In addition, one group received ip two booster doses, and another group 5 booster doses of 1 µg unprecipitated MTV-O protein. The first booster injection was given at one week after the primary immunization; thereafter, the injections were given with two-weekly intervals. The control animals did not receive any treatment. The results are summarized in table 35.

Since in these experiments the animals were subjected to forced breeding at a later age (37 weeks) than in the other experiments, the tumour age was considerably higher. The results clearly demonstrate that a single immunization with 10 µg MTV-O protein precipitated on alum causes a considerable decrease in primary tumour incidence ($p < 0.005$). Booster doses have no favourable effect.

At various intervals after vaccination all animals of the experimental groups were bled from the retro-orbital plexus; the sera of each group were pooled and tested for anti-mammary tumour cell antibodies by membrane immunofluorescence. The results, demonstrated on figure 16, indicate that antibody titer increases as a result of booster injections.

Table 35

INFLUENCE OF INJECTION OF 10 μ g MTV-O PROTEIN VACCINE PRECIPITATED ON ALUM, WITH OR WITHOUT ADDITIONAL BOOSTER DOSES, ON PRIMARY MAMMARY TUMOUR DEVELOPMENT IN BALB/cfc3H

Treatment	tumour incidence	latent period (weeks) \pm SE ^a
none	10/10 (100 %)	56.1 \pm 0.9
10 μ g MTV-O protein and alum	5/13 (39 %)	55.2 \pm 3.2
10 μ g MTV-O protein and alum; 2 booster doses of 1 μ g MTV-O protein	8/18 (44 %)	53.3 \pm 1.5
10 μ g MTV-O protein and alum; 5 booster doses of 1 μ g MTV-O protein	5/ 9 (56 %)	52.6 \pm 0.2

^a: standard error

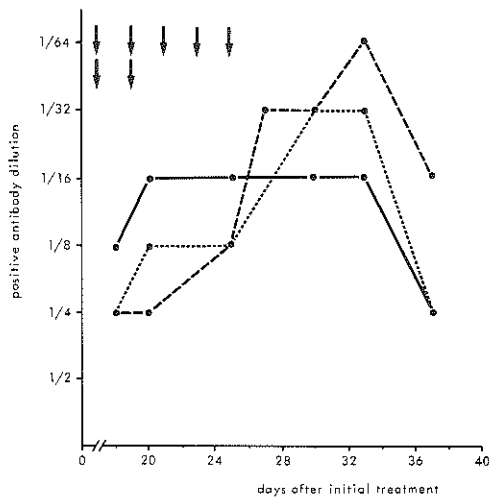


Figure 16:
Anti-MTV antibody levels in BALB/cfC3H mice after different vaccination schedules

Anti-MTV antibodies were established by means of membrane immunofluorescence on MTV-positive cells. RLV-infected cells served as a specificity control. The results were obtained with pools of sera derived from at least 9 animals. All mice received a sc injection with 10 μ g MTV-O protein precipitated on alum.

—: mice received no further treatment;: mice received two additional booster doses of 1 μ g unprecipitated MTV-O protein fraction; -----: mice received five additional booster doses of 1 μ g unprecipitated MTV-O protein fraction. Arrows indicate times of booster doses.

7.3. Discussion

In the high mammary cancer strains GR and BALB/cfC3H immunization with a dose of 10 μ g MTV-S protein vaccine induces a delay in primary tumour development of 9.0 and 7.5 weeks, respectively. For the GR strain, this is in accord with earlier findings using live virus (Van der Gugten and Bentvelzen, 1969). However, in the C3Hf strain, which normally produces tumours at a moderate incidence and at a late age, vaccination leads to significant acceleration of 19.2 weeks in tumour appearance. In the BALB/c strain, another low MTV expressor, tumours also appeared considerably earlier (10.5 weeks).

Since the BALB/c and BALB/cfC3H strains are very close-

ly related genetically, the different response to vaccination is probably due to the virological status. It seems likely that, only in the case of abundant and early virus production by the recipients, vaccination can result in a delay in primary tumour development. The induction of delay in tumour development in the GR strain by a MTV-S vaccine has been ascribed to antigenic differences between the immunogen and the endogenous MTV-P (Van der Gugten and Bentvelzen, 1969). By means of competitive radioimmunoassay it has recently been demonstrated that indeed MTV-S and MTV-P are antigenically distinct (Teramoto et al., 1977). However, since BALB/cfC3H mice carry the exogenous MTV-S, the effect of the MTV-S protein vaccine in this strain cannot be ascribed to immunological differences between MTV strains.

The MTV-O protein fraction precipitated on alum was highly protective in BALB/cfC3H mice. Like in the vaccination studies on the induction of transplantation resistance (Chapter VI), no favourable effect of booster doses could be observed.

In the Winn test, spleen cells of GR mice treated with 10 µg MTV-S protein fraction caused prevention of the growth of admixed tumour cells, whereas cells of BALB/c mice that had received the same treatment only induced a delay of growth of admixed tumour cells. GR peritoneal cells also showed a higher and longer-lasting reactivity in the direct LAI assay than the cells of BALB/c mice. It seems that good cellular immune reactivity is associated with a delay in tumour appearance. The finding that T cell-enriched cell populations are somewhat more active in inhibiting the growth of tumour cells than unfractionated spleen cells *in vivo* is in agreement with the *in vitro* findings that the MTV directed cellular immune reactivity is T cell dependent (Chapter IV).

After vaccination, mice of the GR strain also developed somewhat higher antibody titers than mice of the BALB/c strain. BALB/cfC3H mice treated with the MTV-O protein

fraction precipitated on alum developed moderate levels of antibodies; after boosting, however, the antibody titers increased, which was not correlated with a better protection. This phenomenon might be explained by the induction of antigen-antibody complexes (Chapter V).

From these studies it became evident that transplantation resistance is not a very sensitive indicator for prophylaxis against spontaneous tumour development. There is another discrepancy from these results and the earlier findings using the same MTV-S protein fraction (Chapter VI) that makes the use of transplantation resistance as a measure questionable: in these earlier studies 1 μ g of the protein fraction in combination with alum was protective against a subsequent tumour challenge in BALB/c mice, 10 μ g and alum had no clear effect, and higher doses caused enhancement of the transplanted tumours. A dose of 10 μ g without any adjuvant can be considered as rather low; nevertheless it causes clear enhancement when primary tumour development is studied. An obvious difference between these vaccination experiments is the time between vaccination and tumour development; the length of this latency period is likely to influence results of vaccination studies.

This time factor may be important for the immune status at the time of tumour development. It is possible that the cellular immune response to MTV induced by the vaccination is transient, like in nontumorous mice of high cancer strains (Gilette and Lowery, 1976) and in tumour bearing mice (Chapter III). Mice of the GR and BALB/cfC3H strains may benefit from the induced temporary cellular reactivity delaying tumour development. If, however, vaccination also induces a persistent antiviral response at the humoral level, this may result in the continuous production of blocking factors, which affect the specific as well as the aspecific cellular immune response, thus resulting in enhanced tumour development in strains with a very long latent period.

CHAPTER VIII

GENERAL DISCUSSION

In view of the existence of natural cellular and humoral MTV-specific immune responses in various categories of healthy mice, the choice of "normal" (i.e. unresponsive) control animals remains a problem in immunological studies. For the studies concerning the reproducibility of immunologic assays (Chapter II), the immunologic events during tumour growth (Chapter III and V) and the role of spleen cell subpopulations in MTV-directed cellular immunity (Chapter IV) there was a need for absolutely unresponsive normal control animals. Therefore, in these studies we used 6-8-week-old males. To evaluate the immunologic effect of a given vaccination scheme, it was necessary to use female age-matched (8-15 weeks old) control animals in these experiments (Chapter VI and VII). We never found specific anti-MTV immune responses in our normal control animals. Ihle et al. (1976) using the RIA, report that pooled sera from 6 to 12-week-old GR males are negative, whereas GR females are positive. Pooled sera from female 8 to 12-week-old BALB/cfC3H mice were positive only in a low dilution; the low MTV-expressors developed antibodies at a very much later age. In view of these results, it is not surprising that our normal control animals all gave negative results with the far less sensitive membrane immunofluorescence method.

Except for Blair's group (Blair, 1976), most studies on cellular anti-MTV reactions revealed that before tumour development lymphoid cells of high MTV-expressors do not respond to MTV, whereas cells of low MTV expressors sometimes yield positive results (Lopez et al., 1976; Sigel et al., 1976; Gillette and Lowery, 1976).

As a low MTV expressor, we mostly used BALB/c mice. The aforementioned investigations are mostly performed with the

American BALB/c subline, which seems to show less expression of the endogenous MTV-O than does the Dutch BALB/c subline (Peter Bentvelzen, Don Fine; personal communication). Since the expression of cellular anti-MTV responses is probably inversely related to MTV-expression, it is no surprise that we did not find cellular anti-MTV responses in our healthy BALB/c mice.

A highly interesting subject in tumour immunology is the immune status in animals just before the tumour becomes clinically detectable. However, such an investigation requires extremely time-consuming longitudinal studies on mice which may develop a primary tumour within a relatively short period of time. Also, the need for great quantities of lymphoid cells in cellular immunological assays makes such an approach technically almost impossible. The study of suppressive immunological factors during tumour growth may, however, provide some insight into the insufficiency of the immune response with regard to tumour prophylaxis.

The higher MTV expression which occurs during ageing is related with higher antibody titers (Ihle et al., 1976), whereas the cellular immune response already displays unresponsiveness before the tumour becomes clinically detectable. This unresponsiveness may possibly be induced by slight but persistent levels of soluble antigen-antibody complexes, which are known to inhibit the cellular immune response (Gorczyński et al., 1975), whereas B cells can be inhibited only by immobilized immune complexes (Ryan et al., 1975).

As has been shown in this investigation (Chapter IV) antigen-antibody complexes have a direct suppressive effect on the cellular immune system. In addition they may evoke unresponsiveness in an indirect manner: it has been shown that antibody of the IgG₁ class, on forming a complex with antigen, can induce a long-lasting type of tolerance, apparently mediated by suppressor T cells specific for the antigen. These suppressor T cells may affect either T or B cells (Waksman, 1977).

The situation in MTV-infected mice seems to mimic the chronic infection with lymphocyte choriomeningitis virus in mice: the latter do not express cellular reactivity to the virus (Citrak and Lehmann-Grube, 1974), but virus-antivirus immune complex deposits have been found in the renal glomeruli (Olding et al., 1976).

The present investigation confirms the results of other authors on the presence of anti-MTV antibodies in tumour bearing mice; humoral reactivity was higher in the high-MTV-expressing BALB/cfC3H strain than in the low MTV expressor BALB/c. The finding that unresponsiveness at the cellular level is abolished when the tumour develops is also in accordance with earlier findings (Lopez et al., 1976). Unresponsiveness may wane because either new receptors or new cells are generated, or because excess antigen paralyzes a suppressive T cell system.

In this investigation, a good agreement was found between reactivity as measured by the LAI, the LS and the Winn assays; this indicates that both LAI and LS reactivity may be trustworthy *in vitro* parameters for the *in vivo* cellular immune response.

Anti-MTV reactivity was found to be T cell-dependent. In addition, we found that adherent cells play a crucial role: in the LAI-test (and probably also in the LS test), they provide the first signals for reactivity to MTV.

A new aspect that arises from this study is the observation that cellular immunity declines sharply when the tumour reaches a weight above approximately 2 grams, but recovers partly when tumour growth continues. We found three suppressive mechanisms that all exert their influence very early after tumour development: (i) adherent suppressor cells; (ii) blocking factors in the serum; and (iii) an inhibitory substance covering the spleen cells. Evidence was presented that the two latter factors are antigen-antibody complexes. In addition to those factors, other

authors have provided evidence for the existence of T suppressor cells (Roubinian et al., 1976).

Thus, the following immunologic events are likely to occur: When the tumour starts to develop, only low levels of antigen-antibody complexes will be present. Antigen levels increase steeply just before the development of the tumour (Ritzi et al., 1976); this results in the adequate triggering of lymphoid cells. This causes a relatively good cellular immune response when the tumour is still small. However, the antibodies in the circulation bind to the tumour cells and subsequently capping and shedding of the immune complexes takes place. Gradually, these complexes will be found in the entire circulation; they will bind to the lymphoid cells and thus make them nonresponsive. New populations of cells that are generated may be the cause of the oscillation in reactivity seen after the tumour has become heavier than 3 grams; however, the original level of reactivity is never reached again. It must be assumed that the new populations of lymphoid cells are confronted with such an overwhelming amount of antigen-antibody complexes that the corresponding receptors and lymphokine acceptor sites are almost immediately covered. For this reason, they cannot become adequately stimulated.

It is a reasonable assumption that these events will also affect the humoral immune response. We found a clear oscillation in antibody titer during tumour growth. This is in accord with the findings of Müller et al. (1971), who also found sera of some tumour bearing GR mice to be negative for antiviral antibodies; in case the sera were positive, a higher titer than in normal animals was found. However, the methods used (membrane immunofluorescence and the micro-Ouchterlony assays, respectively) are not very sensitive. Ihle et al. (1976) do not mention variation in antibody titer in tumour bearing animals, but they always tested pools of sera obtained from several mice. In

cooperation with the Frederick Cancer Research Centre, we tested some individual tumour bearing mice repeatedly during 2 months by means of the RIA and membrane immunofluorescence assays; preliminary results indicate that the antibody titer as measured by RIA in individual tumour bearing mice is variable; some sera yielded even negative results.

The oscillation in antibody titer observed in a longitudinal study in tumour bearing animals may be due to the inactivation of helper T cells of the B cell system by soluble antigen-antibody complexes (Waksman, 1977). Precursor B cells are also known to become nonresponsive when confronted with large amounts of antigen (Desaynard and Waldmann, 1976). Mature B cells have been found to be inhibited only by insolubilized immune complexes (Ryan et al., 1975), but it is possible that the complexes aggregated on other lymphoid cells will serve as such inhibitors. When the blood passes the kidney, lymphocytes will also be confronted with the immune complex deposits in the renal glomeruli, which may exert their inactivating influence on the mature lymphocytes.

Antigen-antibody complexes may also be the cause of the inhibition of humoral cytotoxic anti-MTV responses, which occurs at low dilutions in sera from tumour bearing mice (Stolfi et al., 1977).

In human breast cancer patients, the cellular immune response was extensively studied by the group of Grosser and Thompson by means of the LAI assay (1975). As an antigen, they used KCl extracts from malignant breast tumours. They observed that stage I breast cancer patients showed positive cellular immune responses to breast cancer antigens, whereas in patients with disseminated disease immune reactivity is generally impaired. The decrease in reactivity in late stage breast cancer patients has also been observed when the migration inhibition (Black et al., 1974; Cochran et al., 1974) and the mixed lymphocyte

culture (Blomgren and Baral, 1977) tests were used. With the LAI assay, it was demonstrated that reactivity of the lymphoid cells in breast cancer patients with decreased reactivity was increased after slight trypsinization (Grosser and Thompson, 1976), which mimics our results in mice bearing mammary tumours.

It has been found that the erythrocyte receptor sites of T lymphocytes from breast cancer patients are masked; this could be reversed by papain treatment (Whitehead et al., 1976). As in the mouse system, cellular immune reactivity could be inhibited by factors in the serum of human breast cancer patients; this has been demonstrated by means of the LAI (Flores et al., 1977) and the migration inhibition (Rieche et al., 1976) assay. Pleural effusions from breast cancer patients contain complexes of breast-cancer-associated antigen and corresponding antibodies (Gorsky et al., 1977). Also tumour antigen has been detected in their serum (Flores et al., 1977). In addition, an antigen that reacted to gp52 of the murine MTV has been demonstrated on lymphocytes from human breast cancer patients (Wiseman et al., 1977).

In view of the fact that human breast cancer cells show capping of antigens upon incubation with antiserum and subsequent shedding of these antigen-antibody complexes (Nordquist et al., 1977), it is likely that the immunologic events that take place during tumour growth in the human and the mouse system are basically the same.

Mice are able to respond immunologically to MTV, but this response is insufficient to prevent the development of a tumour. Therefore, it was investigated whether tumour prophylaxis could be obtained by increasing the anti-MTV immune response before tumour development. Since by vaccination with whole virus potentially oncogenic material would be introduced into the host, the seemingly best approach would be the use of purified viral proteins, which are expressed on the tumour cell membrane. However, in 1975, when

the vaccination studies were started, purified gp52 was not available; therefore, vaccination studies were performed with an MTV-derived protein fraction which contained approximately 60 % gp52.

Since studying the influence of vaccination on primary tumour development is very time-consuming, the majority of our vaccination experiments have been carried out by measuring the induction of resistance to transplanted mammary tumours. From our results, four main conclusions can be drawn:

(i) mouse strain differences influence the result of a given vaccination procedure; besides from immune response gene differences, this probably results also from a different MTV-expression, and subsequently, a different status of natural immune response to MTV;

(ii) there is a clear dose-dependency, in that a low dose of antigen will tend to give protection, and a high dose results in enhancement of tumour development;

(iii) structure of the antigen is important;

(iv) tumour enhancement after vaccination is associated with the induction of factors in the serum that block the cellular immune response by the vaccination procedure.

Our preliminary results indicate that vaccination with purified gp52 so far does not seem to have a different effect than the administration of the aforementioned crude preparations.

It is highly important to devise vaccination procedures which circumvent the induction of blocking factors. It will be mandatory to stimulate only the cellular immune response, and to keep antibody formation as low as possible. Induction of cellular immunity depends on the structure and hydrophobicity of the antigen (Wilkinson, 1976); we therefore will study the possibility of alteration of the antigen by conjugating lipid to purified gp52, a procedure that is known to enhance cellular immune responses (Hunter and Strickland, 1975). Non-virion, but virus-coded TSSA('s) are expressed on the membrane of mammary tumour cells. We are

presently investigating whether such proteins exist in the MTV-system. When it is possible to purify them, they might be suitable for vaccination against mammary tumour development.

The majority of authors who studied cellular and humoral anti-breast-cancer immunity in patients observed that a certain proportion of healthy women also show positive reactions. Apparently, natural anti-breast-cancer responses also occur in the human species. It is therefore not excluded that specific or nonspecific immune stimulation involves the risk of tumour enhancement. Until now no tumour enhancement after immune therapy in breast-cancer-patients has been reported (Nathanson, 1977). However, it remains an open question whether the clinical trials have been sufficiently accurate to establish such effects: it is unavoidable that groups of patients are rather heterogenous, and this may obscure a tumour-enhancing effect after immune therapy in individual patients.

When no method is found to prevent the induction of blocking factors, vaccination in humans, as well as immune therapy, will become considerably complicated. For both procedures, it will become necessary to establish first under which conditions of anti-breast cancer immune response, immune manipulations may give favourable results. Such experiments on vaccination will be, in view of the anticipated long-latency period of breast cancer, extremely long-lasting. The need to carefully monitor natural immune responses before vaccination will limit for practical reasons this procedure to high-risk groups only. Since results of clinical trials on specific or nonspecific immune stimulation in patients are expected to become available within a reasonable period of time, only immune therapy will then have realistic perspectives.

SUMMARY

The mouse mammary tumour virus (MTV) is horizontally and vertically transmitted in the mouse. MTV induces mammary tumours and structural MTV proteins are expressed on the tumour cell membranes. We investigated the immunological events during tumour growth. In addition, it was studied whether protection against growth of transplanted tumours or primary tumour development could be induced by treatment with an MTV protein vaccine.

For our studies, we used the high MTV expressing GR and BALB/cfC3H and the low MTV-expressing BALB/c, C3Hf and DBA/2f strains. In all strains, MTV is expressed long before tumour development; at that time, the mice also show anti-MTV immune responses. Humoral responses are higher in the high-MTV expressors, but cellular responses are higher in the low MTV expressors. The cellular unresponsiveness in the high MTV expressors is broken when a tumour transplant is given. During tumour growth, both cellular and humoral immune responses become higher than they were previously. The immunologic events during tumour growth, including immune suppressive mechanisms, have not been extensively investigated in the MTV-system.

Chapter II describes the immunological techniques that were applied to establish the immune response.

The presence of anti-MTV antibodies was established with membrane immunofluorescence to specific target cells; embryonic fibroblasts were used as a control. Cellular immunity was investigated with the direct leukocyte adherence inhibition (LAI) assay; this test is based on the fact that the number of cells that adhere to plastic or glass surfaces is reduced, when antigen is added. We also used the leukocyte stimulation (LS) test, in which ^{14}C -thymidine incorporation is taken as a measure for cell

proliferation in the presence of antigen. In both assays, Rauscher leukaemia virus (RLV) was used as a specificity control.

To determine which cells were necessary for the MTV-specific LAI-reaction, we developed the indirect LAI-assay. This assay proved to be more sensitive than the direct LAI-assay. The indirect assay involves the transfer of the leukocyte adherence inhibition factor, (LAIF) which is produced by spleen cells from immune animals when they are cultured with antigen, to indicator cells; for the latter, peritoneal exudate cells from normal mice were used. Since there is no previous experience with the LAI-assay in the MTV-system, the specificity of the direct and the indirect LAI assays has been extensively established.

Chapter III describes an investigation to the anti-MTV immune response during spontaneous tumour growth. For this purpose, the immune response in BALB/c, BALB/cfC3H and GR mice bearing tumours of varying sizes were used. Specific humoral immunity was found to be present on most occasions, but antibody titer fluctuated during tumour growth. In both the LS and direct LAI assays, anti-MTV reactivity peaked at a tumour weight of about one gram; afterwards, it almost completely disappeared. Reactivity increased again to about half of the original level when the tumour reached a weight of about three grams. Peak proliferation of lymph node cells was observed at a tumour weight somewhat higher than when peak proliferation of spleen cells occurred.

Chapter IV describes a study in which the role of different spleen cell subpopulations in MTV directed cellular reactivity was investigated. Spleen cells from immunized mice were treated with crude separation techniques prior to testing. It was established that the lymphocyte population which responded *in vitro* with proliferation in the LS assay in the presence of MTV were T cells. For the reaction in the LAI assay, both T cells and cells with adherent proper-

ties are necessary. Cell-to-cell communication is mediated by soluble factors. It became established that, for the production of LAIF, the following sequence of incubation between T- and adherent cells is required:

(1) incubation of adherent cells with MTV; (2) transfer of a soluble factor, SF_1 , produced by the adherent cells to T cells; (3) transfer of another soluble factor, SF_2 , released by T cells to the adherent cells. Upon this contact the adherent cells release LAIF.

Chapter V describes an investigation to the cause of the decline in cellular immune reactivity, that is observed during tumour growth. Three mechanisms that inhibited the MTV-specific immune response were observed:

(i) the depression in MTV-specific proliferation was partly due to the presence of suppressor cells which have adherent properties. These suppressor cells occur early during tumour growth and exert their greatest influence when the tumour weighs 0.5 - 1.5 grams. In animals bearing larger tumours, the suppressor cell activity was lower.

(ii) Reactivity of nonresponsive T-cell-enriched cell populations from animals bearing large tumours could be restored by mild trypsinization or extensive washing of the cells. When cells from mice immunized with MTV were incubated with the wash fluid of the initially unresponsive cells, MTV-specific proliferation and LAI reactivity was inhibited. Washings of normal cells had no such effect.

(iii) Sera from all tumour bearing animals tested contained factors that block cellular reactivity.

The two latter factors appeared early after tumour development. They both contained MTV and anti-MTV antibodies, whereas wash fluid from lymphoid cells and sera from normal animals were negative. These results make it likely that the paralysis of the immunocytes is caused by excess of immune complexes.

Chapter VI describes vaccination experiments, in which the induction of transplantation resistance against MTV-containing neoplasms by treatment with a MTV-protein fraction that consisted for approximately 60 % of the major viral membrane glycoprotein gp52 was studied. The vaccine was derived from MTV-S. BALB/c and DBAf mice were vaccinated with different doses of this vaccine. Twenty days after vaccination, the mice were challenged with live tumour cells: BALB/c with an MTV-O-induced mammary tumour, DBAf with the MTV-positive leukaemia L1210. Alum and an interphase material isolated from *Mycobacterium smegmatis* (IPM) were used as an adjuvant; control animals were treated with the adjuvant alone.

In BALB/c mice treated with 1 µg of the gp52-enriched protein fraction precipitated on alum, tumour development was delayed 10 weeks; a dose of 10 µg had no clear effect. A dose of 100 µg, however, caused clear acceleration of tumour development: each of the vaccinated animals developed a tumour, as compared to 60 % of the control group.

In DBAf mice low doses of the vaccine precipitated on alum accelerated tumour development. In general, acceleration was stronger when a higher dose was used.

Fifty µg of IPM and different doses of the gp52-enriched protein fraction in DBAf mice resulted in protection. In combination with 100 µg IPM, however, tumour development was accelerated.

Specific cellular immune reactivity to MTV was determined by means of the direct LAI assay. Serum was tested for the presence of factors that either block or enhance the indirect LAI reaction; the presence of anti-MTV antibodies was established with the membrane immunofluorescence test. Protection was observed when serum blocking factors were absent, even if cellular immune reactivity and antibody titer were low. When serum blocking factors were present, protection was observed only when it was combined with high antibody titers and strong cellular immunity.

In Chapter VII, the influence of the gp52-enriched vaccine on primary tumour development is described. Subcutaneous inoculation of a dose of 10 µg of MTV-S derived vaccine induced a significant delay in the appearance of primary mammary tumours in the high cancer strains GR and BALB/cfC3H; in the strains BALB/c and C3Hf, however, which have a moderate tumour incidence at an advanced age, this treatment resulted in a slight and substantial acceleration respectively, of primary tumour development. The induced specific cellular immune reactivity after vaccination as measured with the *in vivo* Winn-test and the *in vitro* LAI assay was strongest in the GR-strain as compared to the BALB/c strain. The titer of antibodies to tumour cells as estimated by membrane immunofluorescence was also higher in the GR strain.

In BALB/cfC3H mice the influence of different vaccination schemes with an MTV-O derived protein vaccine on primary tumour development was studied. Before subcutaneous injection the vaccine was precipitated on alum. A dose of 10 µg of this vaccine resulted in a 61 % decrease in tumour incidence. Two or five additional booster injections with 1 µg of the unprecipitated protein vaccine had no beneficial effect, although the amount of antibodies measured was increased after boosting.

In the general discussion (Chapter VIII) are discussed the immune state of the mice before and after the development of an MTV-induced mammary tumour, and the problems in achieving successful vaccination. The literature concerning the immune state in humans to breast cancer antigens is summarized. Many immunological aspects in man and mice show a striking similarity. Therefore, when immune therapy and vaccination in humans is taken into consideration, one has to pay attention to the findings in the mouse system.

SAMENVATTING

In muizen wordt kanker van de melkklier veroorzaakt door het mammary tumour virus (MTV); dit virus wordt voornamelijk overbracht op de nakomelingen via de melk en/of in de vorm van genetische informatie. Eiwitten die deel uitmaken van het MTV worden ook afgezet op de wand van de tumorcel.

Wij onderzochten de anti-MTV immuunrespons gedurende de tumorgroei. Tevens werden vaccinatie-experimenten uitgevoerd met een MTV-eiwitfractie; getracht werd resistentie op te wekken tegen getransplanteerde en spontane tumoren. Voor dit onderzoek gebruikten wij de GR- en de BALB/cfC3H-stammen, die een sterke MTV expressie en een hoge tumorincidentie hebben, alsmede de BALB/c-, C3Hf- en DBAf-stammen, die slechts geringe hoeveelheden MTV produceren en waarbij de tumorincidentie laag is.

In al deze muizenstammen wordt al MTV in het serum aangetroffen voordat er een tumor aanwezig is; de muizen vertonen dan een positieve immuunreactie tegen MTV. De humorale respons is hoger in dieren die een sterke MTV expressie hebben, maar de cellulaire reactie is groter in muizen met een zwakke MTV-expressie. Transplanteert men echter in stammen met een sterke MTV-expressie een tumor, dan vertonen de muizen daarna wel een cellulaire anti-MTV reactie. Als er een tumor aanwezig is wordt de cellulaire en humorale immuunrespons in alle stammen hoger dan zij daarvoor geweest is. Over het verloop van immuunrespons tijdens de tumorgroei en het optreden van immunosuppressieve mechanismen is in het MTV systeem nog weinig bekend.

Hoofdstuk II geeft een overzicht van de technieken waarmee de anti-MTV immuunrespons in dit onderzoek werd bepaald. De aanwezigheid van anti-MTV antilichamen werd bepaald met de indirecte membraan immunofluorescentie test; hierbij werden tumorcellen als specifieke targetcellen gebruikt en

embryonale fibroblasten deden dienst als controlecellen. De cellulaire immuniteit werd bepaald met de directe leukocyt adherentie inhibitie (LAI) test; deze test is gebaseerd op het principe dat het aantal cellen dat zich vasthecht aan glas of plastic specifiek verminderd is als er antigeen wordt toegevoegd. Tevens gebruikten wij de leukocyt stimulatie (LS) test, waarbij de incorporatie van ^{14}C -thymidine in het cellulaire DNA als maat wordt genomen voor de mate van celproliferatie in de aanwezigheid van een antigeen. In beide testen werd als specificiteitscontrole Rauscher leukemie virus (RLV) gebruikt.

Om te kunnen bepalen welke celsubpopulaties een rol speelden bij de cellulaire anti-MTV immuun respons werd de indirecte LAI test ontwikkeld. Deze test bleek bovendien gevoeliger te zijn dan de directe methode. In de indirecte test wordt de leukocyt adherentie inhibitie factor (LAIF), die door immune miltcellen in aanwezigheid van antigeen wordt geproduceerd, overgebracht op indicatorcellen; hiervoor werden peritoneaalcellen van normale muizen gebruikt. Aangezien de LAI-test nog niet eerder in het MTV-systeem gebruikt is, hebben wij de specificiteit van de directe en de uitgebreid onderzocht.

Hoofdstuk III beschrijft de resultaten van een onderzoek naar de relatie tussen de tumorgroei en de expressie van de anti-MTV immuun respons. Als proefdier werden BALB/c-, BALB/cfC3H- en GR-muizen met een spontane melkkliertumor gebruikt. Meestal waren er specifieke anti-MTV antilichamen in het serum aanwezig, maar de antilichaamtiter fluctueerde gedurende de groei van de tumor. De cellulaire immuniteit tegen MTV, die met de directe LAI en de LS test werd vastgesteld was het hoogste als de tumor een gewicht had van ongeveer 1 gram; daarna verdween de reactiviteit bijna geheel. Als de tumor een gewicht van drie tot vier gram had bereikt, herstelde de cellulaire immuniteit zich gedeeltematig. Lymfkliercellen bereikten de hoogste proliferatie bij een iets hoger tumorgewicht dan miltcellen.

Hoofdstuk IV beschrijft een onderzoek, waarin werd nagegaan welke subpopulatie van de lymfoïde cellen verantwoordelijk was voor de cellulaire anti-MTV reactiviteit. Hiertoe werden miltcellen van geïmmuniseerde dieren voor het uitvoeren van de test behandeld met celscheidingstechnieken. In de LS test bleken alleen de T-cellen te prolifereren na incubatie met MTV. Voor de MTV-specifieke LAIF productie waren zowel T cellen als cellen met adherente eigenschappen noodzakelijk. De communicatie tussen deze celpopulaties werd tot stand gebracht door oplosbare factoren. Nader onderzoek wees uit dat aan de LAIF-productie waarschijnlijk de volgende reeks gebeurtenissen vooraf gaat: (1) als cellen met adherente eigenschappen met MTV geïncubeerd worden, produceren zij een oplosbare factor, SF_1 ; (2) als SF_1 in contact komt met T cellen, produceren deze laatsten een andere oplosbare factor, SF_2 ; (3) als SF_2 op cellen met adherente eigenschappen wordt overgebracht, produceren deze LAIF.

Hoofdstuk V beschrijft een onderzoek naar de oorzaken van de verminderde cellulaire reactiviteit, die gedurende de tumorgroei optreedt. Er werden drie suppressorfactoren gevonden die de cellulaire anti-MTV reactiviteit gedurende de tumorgroei remmen:

(1) de depressie in MTV-specifieke celproliferatie werd gedeeltelijk veroorzaakt door de aanwezigheid van suppressorcellen met adherente eigenschappen. Deze suppressorcellen zijn reeds aanwezig als de tumor nog klein is; hun grootste invloed werd gemeten bij een tumorgewicht van 0,5 - 1,5 gram. In dieren met grotere tumoren was de invloed van de suppressorcellen minder.

(2) T-cel verrijkte subpopulaties van muizen met grote tumoren, die niet positief waren in de LS test, vertoonden een MTV-specifieke reactie nadat zij erg vaak gewassen waren of een milde trypsinisatieprocedure hadden ondergaan. De wasvloeistof van de niet-responderende cellen van tumordragers remde de MTV-specifieke LAIF-productie en LS-

activiteit van immune cellen na preïncubatie.

(3) De sera van alle tumordragende dieren die getest werden, bleken factoren te bevatten die de cellulaire immuniteit tegen MTV remmen.

De inhiberende werking van serum en wasvloei-stof van lymfoïde cellen van tumordragers werd reeds bij dieren met kleine tumoren aangetroffen. Beiden bleken zowel MTV als anti-MTV antilichamen te bevatten; bij serum en wasvloei-stof van lymfoïde cellen van dieren zonder tumor, die niet remmend werkten, was dit niet het geval. Deze resultaten maken het aannemelijk dat de lymfoïde cellen geïnhibeerd worden door een overmaat aan antigeen-antilichaam complexen.

Hoofdstuk VI beschrijft de resultaten van vaccinatie experimenten, waarbij als vaccin een MTV-eiwitfractie, die voor ongeveer 60 % uit het grootste virale membraan eiwit, gp52, bestond, gebruikt werd. Het effect van de vaccinatieprocedures werd getest door de resistentie tegen getransplanteerde tumoren te meten. Twintig dagen na vaccinatie werden gevaccineerde BALB/c en DBAf muizen ingespoten met respectievelijk een door MTV-O geïnduceerde melkkliertumor en de MTV-positieve L1210 leukemie. Als adjuvant werd aluin gebruikt, en een stof die geïsoleerd was uit Mycobacterium segmatis (IPM).

In BALB/c-muizen die met 1 µg van de op aluin geprecipiteerde MTV-fractie gevaccineerd waren, was de tumoropkomst 10 weken vertraagd; een dosis van 10 µg had geen duidelijk effect op de groei van de getransplanteerde tumoren. Een dosis van 100 µg veroorzaakte echter een duidelijke versnelling van de tumoropkomst: 100 % van de gevaccineerde, doch slechts 60 % van de controledieren ontwikkelden een tumor binnen de observatieperiode. In DBAf-muizen veroorzaakte lage doses van het op aluin geprecipiteerde vaccin reeds verkorting van de latentietijd; over het algemeen was deze versnelling sterker naarmate de dosis die voor vaccinatie gebruikt werd, hoger was.

Als DBAf muizen gevaccineerd werden met 50 µg IPM in combinatie met verschillende doses van de MTV-eiwit fractie was de tumorontwikkeling vertraagd. Gebruikte men echter 100 µg IPM, dan was de tumorgroei versneld.

Specifieke cellulaire anti-MTV reactiviteit en de aanwezigheid van blokkerende factoren in het serum na vaccinatie werd vastgesteld met de LAI test. Anti-MTV antilichamen werden bepaald met de membraan immunofluorescentie test. De afwezigheid van blokkerende factoren in het serum was gecorreleerd met protectie tegen tumorontwikkeling, zelfs als de cellulaire en humorale anti-MTV reacties erg laag waren. Waren blokkerende serumfactoren wel aanwezig, dan trad protectie alleen op indien dit gecombineerd was met hoge cellulaire reactiviteit en met hoge antilichamtiters.

Hoofdstuk VII beschrijft een onderzoek waarbij de invloed van het voor gp52 verrijkte vaccin getest werd op de ontwikkeling van spontane melkkliertumoren. In de BALB/cfC3H- en de GR-stam, die beiden een hoge tumorincidentie hebben, veroorzaakte subcutane injectie van 10 µg van een van MTV-S afkomstig vaccin een significante vertraging in tumorontwikkeling. In de BALB/c- en C3Hf-stammen echter, die beiden een lage tumorincidentie hebben, had vaccinatie met dezelfde dosis een versnelling van tumorontwikkeling tot gevolg. Deze versnelling was alleen in de C3Hf-stam significant.

De door de vaccinatie opgewekte MTV-specifieke cellulaire en humorale immuunrespons was hoger in de GR- dan in de BALB/c-stam.

De invloed van verschillende vaccinatieschema's op de ontwikkeling van primaire mammatumoren werd bepaald in de BALB/cfC3H-stam. Hiervoor werd een voor gp52 verrijkt vaccin gebruikt dat afkomstig was van MTV-O. Subcutane injectie van dit vaccin in combinatie met aluin veroorzaakte een vermindering van 61 % in tumorincidentie vergeleken met onbehandelde controle dieren. Twee andere

groepen muizen ontvingen na deze injectie nog respectievelijk 2 en 5 intraperitoneale booster injecties met 1 μ g van het MTV-0 vaccin; deze behandelingen resulteerden echter niet in een sterkere verlaging van de tumorincidentie. Niettemin trad na de booster injecties een verhoging van de antilichaamtiter op.

In de algemene discussie (Hoofdstuk VIII) wordt de immunusstatus bij melkklierkanker in het muizesysteem en de problemen die bij vaccinatie optreden, besproken. Ook worden de literatuurgegevens over de tegen borstkankerantigenen gerichte immunusstatus bij de mens samengevat. Veel immunologische gegevens in beide species vertonen een treffende overeenkomst. Bij het overwegen van immunotherapie en vaccinatie in de humane praktijk dient men derhalve rekening te houden met de bevindingen in het muizesysteem.

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ABBREVIATIONS

ADCC	antibody dependent cellular cytotoxicity
cpm	counts per minute
D	daltons
FA	Freund adjuvant
FCS	Foetal calf serum
FeLV	Feline Leukaemia Virus
FITC	fluorescein isothiocyanate
FOCMA	Feline oncornavirus associated cell membrane antigen
FLV	Friend leukaemia virus
iAp	intracytoplasmatic A particles
ip	intra-peritoneal
IPM	interphase material isolated from <i>Mycobacterium smegmatis</i>
LAI	leukocyte adherence inhibition
LAIF	leukocyte adherence inhibition factor
LS	leukocyte stimulation
MER	derivative of BCG
UV	ultraviolet

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In dit proefschrift wordt beschreven dat het experimentele werk hier onder leiding van Dr. P. Bentvelzen werd verricht.

