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Induction of type II collagen-specific antibody production in blood lymphocyte cultures of Rhesus monkeys (*Macaca mulatta*) with collagen-induced arthritis using the immobilized native antigen

B. A. 'T HART*, C. A. D. BOTMAN & N. P. M. BAKKER *Department of Chronic and Infectious Diseases, ITRI-TNO Institute, Rijswijk, The Netherlands*

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SUMMARY

Peripheral blood mononuclear cells (PBMC) from Rhesus monkeys previously immunized with bovine type II collagen to induce arthritis were cultured with the same antigen. Because the native protein is poorly soluble in culture medium a heating step is often used. The antigen in this form induced PBMC proliferation, but epitopes for the induction of antibody production and arthritis were lost. To keep the native protein intact it was coated on affigel beads. With the immobilized antigen specific antibody production could be induced.

Keywords peripheral blood mononuclear cells type II collagen antibody production

INTRODUCTION

Human rheumatoid arthritis (RA) is often associated with cellular and humoral autoimmunity to type II collagen (CII) (Clague, Shaw & Holt, 1981; Klareskog *et al.*, 1986; Morgan *et al.*, 1989; Tarkowski *et al.*, 1989). The possible role of the autoimmunity to a major constituent of the joint cartilage, however, is only poorly understood. In recent years collagen-induced arthritis (CIA) has been established as an animal model to investigate the mechanism(s) by which experimentally induced (auto)immunity to CII can lead to arthritis (Trentham, 1982).

We have investigated in Rhesus monkeys the different contribution of humoral and cellular immunity to CII to the susceptibility to CIA (Bakker *et al.*, 1990b). In that study we determined the capacity of peripheral blood mononuclear cells (PBMC) from animals immunized with CII of bovine origin (B-CII) to proliferate and produce antibodies in culture with the same antigen. A complicating factor in such studies is that B-CII in its native form is poorly soluble in culture medium and therefore gives unreliable results. Many investigators dissolve the protein in culture medium by heating briefly (e.g. Holmdahl *et al.*, 1985). However, immunization of rats with a heated B-CII solution emulsified in Freund's complete adjuvant induced cellular but not humoral immunity to the native protein (unpublished data, see also Stuart, Townes & Kang, 1981). Neither did it evoke arthritis, indicating that heating destroys B-CII epitopes which are indispensable for the induction of arthritis as well as humoral immunity. Similarly, we could demonstrate the induction of proliferation, but not antibody production in cultures of rhesus monkey PBMC with heated B-CII.

In the present study we have developed a procedure for the induction of specific antibody production in cultures of rhesus monkey PBMC by immobilization of the antigen in its native form.

Correspondence: B. A. 't Hart, Department of Chronic and Infectious Diseases, ITRI-TNO Institute, PO Box 5815, 2280 HV Rijswijk, The Netherlands.

MATERIALS AND METHODS

Animals

All rhesus monkeys were bred and raised at the TNO primate centre in Rijswijk. The animals used for this study had been immunized several times with B-CII (Bakker *et al.*, 1990). The isolation of B-CII from bovine nose septum has been described in the same report. Blood for serum production or for the isolation of PBMC was obtained from immunized monkeys which developed arthritis (susceptible animals: 1KM, M15, D3, L65) or which failed to develop any clinical or serological sign of inflammation (resistant animals: 1RQ, M14).

Isolation and culture of PBMC

The animals were anaesthetized with 0·1 ml/kg of a mixture of 9 volumes ketamine (10 mg/ml; Chassot & Cie Ag, Bern, Switzerland) and 1 volume vetranquil (10 mg/ml acepromazine and 5 mg/ml chlorbutanol; Compagnie Rousselot, Paris, France). Blood was taken in heparinized (thromboliquine) vacutainers (Becton Dickinson, Meylan Cedex, France) and diluted with an equal volume of Hanks' balanced salt solution (HBSS; GIBCO Ltd, Paisley, Scotland). PBMC were isolated by

density gradient centrifugation on lymphocyte separation medium (Organon Technika Corporation, Durham, NC). The cells from the interphase of the gradients (PBMC) were washed twice with HBSS. PBMC were cultured in a humidified atmosphere containing 5% CO_2 in 96-well round-bottomed plates (Greiner Labortechnik, Nuertingen, Germany) at a concentration of 40000 cells/well. The culture medium consisted of carbonate-buffered RPMI 1640 containing 200 mM L-glutamine, 200 U/ml penicillin, 200 U/ml streptomycin and 10% (vol.) heat-inactivated fetal calf serum (all from GIBCO). For stimulation with soluble B-CII the protein was routinely dissolved in RPMI 1640 by heating for 30 min at 90°C. Proliferation was assessed by counting the number of viable cells in each culture. Results were expressed by the stimulation index:

$$SI = \frac{\text{the cell number in stimulated cultures}}{\text{the cell number in a control culture without antigen.}}$$

Coating of affigel beads

Affigel-15 beads (Bio-Rad laboratories, Richmond, CA) contain *N*-hydroxysuccinimide groups which couple to free amino residues in a protein. Usually this will not disturb the conformation of the molecule and hence conformational epitopes will remain intact. For this study the affigel beads were washed thoroughly with 0·05 M acetic acid. Routinely one volume of the washed beads was mixed with one volume of 5 mg B-CII per ml 0·05 M acetic acid for coating. The mixture also contained 0·3 M NaCl to reduce non-specific binding (manufacturer's instruction). When B-CII concentrations < 5 mg/ml were used for coating the beads, the stimulation of antibody production was reduced, while beads coated with higher B-CII concentrations had only a slightly higher stimulatory capacity (not shown). After 2 h incubation at room temperature under gentle stirring the beads were centrifuged. After removal of the supernatant the beads were washed at least five times with phosphate-buffered saline (PBS, pH 7·4). For post-coating the whole procedure was repeated with 1% bovine serum albumin (BSA) in PBS. Beads coated only with BSA functioned as controls in all assays.

ELISA for C-II-specific antibodies in monkey immune sera

The assay was performed exactly as described by 't Hart *et al.* (1990).

Detection of antibody production induced by the beads

Beads recovered from cell cultures were washed once with PBS and then incubated for 1 min with 0·1% Triton X-100 in demineralized water to lyse adherent cells. After the beads had been washed throughly with PBS-0·1% Tween 20 (at least five times) binding of immunoglobulin was determined by incubation for 1 h at 37°C with alkaline-phosphatase-conjugated affinity-purified goat antibodies directed to heavy chains of human IgM or IgG (Tago, Burlingham, CA). After thorough washing with PBS–0·1% Tween 20 the beads were incubated for about 30 min at 37°C with substrate solution. This solution consisted of 0·5 mg *p*-nitro-phenylphosphate (Sigma Chemical Company, St Louis, MO) per ml 0·1 M glycine-NaOH buffer (pH 10·4), containing 1 mM $MgCl_2$ and 1 mM $ZnCl_2$. Colour development was read at 405 nm.

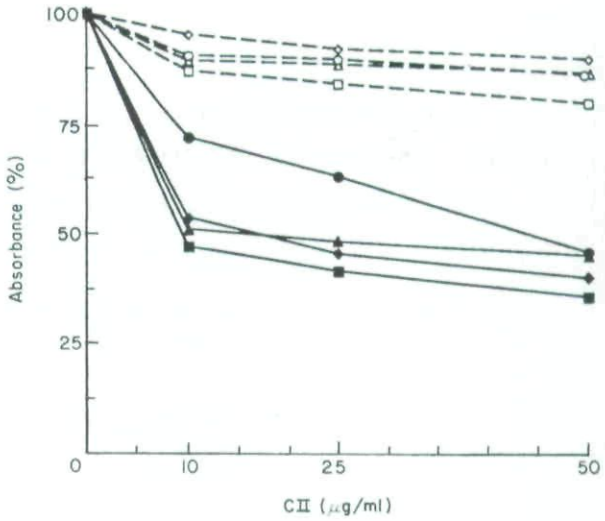

Fig. 1. Destruction of epitopes recognized by IgG antibody by heating of B-CII. Immune sera, diluted 200-fold, of four animals were incubated at 37°C with the concentrations indicated of heat-denatured (30 min heated, open symbols) or native (30 min cold, closed symbols) B-CII. After 60 min incubation the amount of free IgG antibody to native B-CII was determined with ELISA. Results are given as the percentage absorbance (in this ELISA at 405 nm). Results were normalized by defining the measured IgG in sera without B-CII at 100%. □■, 1KM; △▲, 1OX; ○●, L65; ◇◆, M15.

RESULTS

Recognition of native and heated B-CII by monkey immune sera

Immune-sera from four susceptible B-CII-immunized monkeys at 200-fold dilution were pre-incubated with different concentrations of native or heated B-CII. The amount of free IgG antibodies to native B-CII after pre-incubation was determined with ELISA. As shown in Fig. 1, heating considerably reduced the capacity of B-CII to deplete specific IgG from monkey serum. The same experiment was then done with immobilized native B-CII. The results in Fig. 2 show that the B-CII-coated beads effectively removed specific IgG from Rhesus monkey immune sera. Control beads coated with BSA did not remove B-CII-specific IgG. These data show that heating of B-CII destroyed epitopes recognized by specific IgG antibodies, but these remain intact in the immobilized native protein.

Stimulation of PBMC with soluble B-CII

In a first experiment the capacity of different concentrations of heated B-CII to stimulate proliferation and antibody production in cultures of PBMC was determined. As shown in Table 1(a) a low but significant proliferation was observed in cultures of PBMC from the B-CII-immunized animals 1KM and M15. This proliferation was absent in PBMC from the non-immunized animal Z, or from animal TD which had been immunized with an unrelated antigen. No B-CII-specific antibodies were detectable with ELISA in any of the cultures.

In a second experiment PBMC from monkeys 1KM and M15 were again stimulated with different antigen concentrations. In this experiment recombinant IL-2 was added as well, aimed at enhancing the production of B cell activation and maturation factors by B-CII-activated T cells. Despite a strongly increased proliferation (Table 1b) in the supernatants

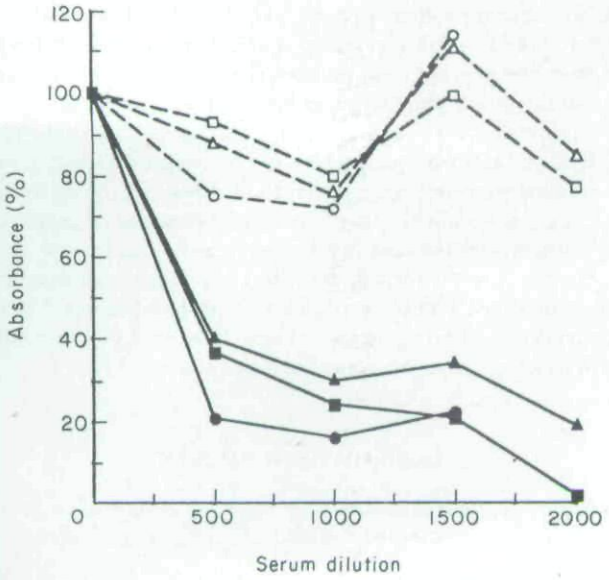

Fig. 2. Depletion of antibodies from rhesus monkey serum with B-CII-coated beads. One volume of B-CII- (closed symbols) or BSA-coated (open symbols) beads (settled bed volume) was incubated at 37 °C with 3 volumes of differently diluted immune sera from three monkeys. After 60 min incubation the amount of free IgG antibodies to B-CII was determined with ELISA. Results are given as the percentage absorbance (in this ELISA at 405 nm). The results from the different animals were normalized by defining the measured IgG in sera without B-CII at 100%. □ ■, L65; △ ▲, 1KM; ○ ●, 1OX.

Table 1. Stimulation of PBMC with soluble C-II

(a) Experiment I	Stimulation index			
C-II (μg/ml)	Z	TD	M15	1KM
0	1·0	1·0	1·0	1·0
2·5	1·1	1·1	1·4	2·3
5·0	0·8	0·5	1·4	2·8
10·0	1·1	0·5	1·8	2·5
20·0	0·8	0·6	2·6	4·3
40·0	0·7	0·3	3·3	6·8

(b) Experiment II	Stimulation index							
	Monkey 1KM rIL-2 (U/ml)				Monkey M15 rIL-2 (U/ml)			
C-II (μg/ml)	—	2	5	10	—	2	5	10
0	1·0	17·5	35·5	38·3	1·0	9·6	12·6	12·8
10	1·5	26·5	42·3	40·5	1·6	15·4	18·4	18·8
20	2·1	32·8	44·8	45·0	1·8	14·8	19·2	19·6
40	1·8	30·3	28·0	41·8	1·8	14·8	17·2	17·0

PBMC were cultured in triplicate with different doses of bovine C-II in the absence or presence (only in experiment II) of recombinant IL-2 that was added at day 0. After 6 days of culture the number of cells per well was counted, and the stimulation index calculated. Donor Z was a non-immune control monkey; donor TD had been immunized with streptococcal cell walls; donors M15 and 1 KM were monkeys immunized with B-CII which had developed CIA (susceptible animals).

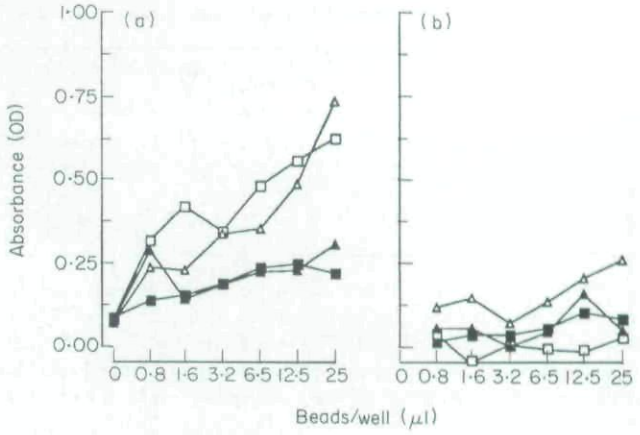

Fig. 3. Induction of antibody production with B-CII-coated beads. PBMC of four monkeys were cultured with different amounts of B-CII- (open symbols) or BSA-coated (closed symbols) beads. Monkeys 1KM and D3 were susceptible to CIA induction (a), while 1RO and M14 were resistant (b). The beads recovered from triplicate cultures were pooled and developed analogous to ELISA plates, as described in Materials and methods. The results show the light absorbance in arbitrary units (OD). (a) △▲, 1KM; □■, D3. (b) △▲, 1RO; □■, M14.

of these cultures, again no B-CII-specific antibodies were detectable with ELISA.

Stimulation of PBMC with immobilized B-CII

PBMC from four B-CII-immunized animals were cultured with B-CII-coated beads. PBMC were obtained from two susceptible (D3 and 1KM) and two resistant animals (1RO and M14). The sera of both groups of animals contain a high amount of B-CII-specific IgG. In this experiment the beads of three identical culture wells were pooled. The results in Fig. 3 show that B-CII-coated beads induced only PBMC from susceptible animals to antibody production, while no antibody production was detectable in cultures with BSA-coated beads. Only antibody molecules binding to the beads were found. Antibodies in the culture medium were not detectable.

This finding was reproduced in three further experiments. Triplicate PBMC cultures were stimulated with 25 μl settled bed volume of B-CII-coated beads in a total culture volume of 150 μl. After culture the beads from each well were developed separately. Again the supernatants of the cultures did not contain B-CII-specific antibodies detectable with ELISA, but development of the beads revealed binding of B-CII-specific antibodies (Table 2).

DISCUSSION

From a group of 14 rhesus monkeys immunized with B-CII 10 developed CIA. These were regarded as susceptible animals. In four animals no CIA could be induced, although these have a high titre of B-CII-specific serum antibodies (Bakker *et al.*, 1990a). The latter animals are therefore regarded as resistant to CIA. Using these two groups of animals the role of the immunity raised against C-II in the course of CIA has been investigated. The results of this study will be published separately (Bakker *et al.*, 1990b). The present report deals with a methodological problem we encountered in that study.

Table 2. Stimulation of PBMC with immobilized C-II

	OD (405 nm)		
Coating	Monkey L65	Monkey 1KM	Monkey M15
BSA-coated beads	98+15	75+12	83+1·9
B-CII-coated beads	397+52	215+10	304+2·3

PBMC from three C-II-immunized susceptible Rhesus monkeys were cultured in triplicate for 8 days with 25 μl affigel-15 beads (settled bed) per 150 μl culture medium. The beads were coated with different concentrations of C-II. After culture the binding of IgM and IgG was determined. Results are expressed in arbitrary units (OD × 10E3; mean + s.d.).

As also shown in Table 1, PBMC from susceptible animals were induced to proliferate in culture with B-CII, although the stimulation remained low. We were unable, however, to detect specific antibody production in these cultures even in the presence of added IL-2. The fact that B-CII is only a weak antigen might, particularly, be explained by the fact that B-CII shows a high degree of structural homology with autologous CII. Hence, the immunological response to B-CII might be suppressed under the influence of tolerance to self-CII. Addition of IL-2 gives a strong enhancement of PBMC proliferation.

Our present results indicate that the heating step, routinely used by many investigators to dissolve B-CII in culture medium, destroys epitopes which are necessary for recognition by the B cell antigen receptor. Analysis with SDS–PAGE showed that the protein molecule remains largely intact (not shown). The destroyed determinants are therefore likely conformational epitopes. Because T cells generally do not recognize conformational epitopes this will not affect the proliferation of this cell type. This explanation is supported by a recent observation in rats that immunization with heat-denatured B-CII induced cellular immunity to the native B-CII, as was tested by delayed type hypersensitivity, but no humoral immunity, as measured by serum antibodies. Moreover, animals immunized with the heated antigen failed to develop arthritis, indicating also that arthritogenic determinants are destroyed.

It has been demonstrated by Logtenberg *et al.* (1986) that PBMC from patients with autoimmune thyroiditis can be induced to produce anti-thyroglobulin antibodies in culture with the autoantigen, when it is coated on the surface of affigel beads. In the present study we have immobilized B-CII in its native form on affigel beads. Our results show that the immobilized native antigen effectively depleted antibodies from monkey immune sera, indicating that epitopes recognized by antibody molecules remained intact. PBMC from susceptible animals were cultured with the B-CII-coated beads or with BSA-coated beads as control antigen. No specific antibodies were found in the culture supernatant, but development of the B-CII-coated beads revealed attached IgG antibodies. In cultures with BSA-coated control beads IgG binding was absent. We conclude therefore that immobilized native B-CII induced specific antibody production in PBMC cultures from susceptible monkeys. We were unable, however, to induce significant antibody production by PBMC obtained from resistant animals. At present we do not have an explanation for this phenomenon. As was shown previously (Bakker *et al.*, 1990b) B-CII-induced PBMC proliferation was also absent in these animals. A likely explanation might thus be that B-CII-reactive T and B cells are not present in the circulation in these animals, or are subjected to suppression. This is the subject of our present research. The absence of antibody production in this resistant group, however, allowed us to conclude that the antibodies binding to the B-CII-coated beads are not plasma antibodies that are passively transferred with the cells, but are produced in the culture.

In our view the method described in this paper enables the study of B cell immunity to different forms of native CII in rheumatoid arthritic patients at the cellular level. Such investigations are planned by us for the near future.

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REFERENCES

BAKKER, N.P.M., VAN ERCK, M.G., ZURCHER, C., FAABER, P., LEMMENS, A., HAZENBERG, M., BONTROP, R.E. & JONKER, M. (1990a) Experimental immune-mediated arthritis in rhesus monkeys. A model for human rheumatoid arthritis? *Rheumatol. Int.* **10,** 21.

BAKKER, N.P.M., VAN ERCK, M.G.M., BOTMAN, C.A.D., JONKER, M. & 'T HART, B.A. (1990b) Collagen-induced arthritis in an outbred group of Rhesus monkeys comprising responder and non-responder animals. Relation between the course of arthritis and collagen-induced immunity. *Arthritis Rheum.* (In press).

CLAGUE, R.B., SHAW, M.J. & HOLT, P.J.L. (1981) Incidence and correlation between serum IgG and IgM antibodies to native type II collagn in patients with chronic inflammatory arthritis. *Ann. rheum. Dis.* **40,** 6.

HOLMDAHL, R., KLARESKOG, L., RUBIN, K., LARSSON, E. & WIGZELL, H. (1985) T lymphocytes in collagen II-induced arthritis in mice. Characterization of arthritogenic collagen II-specific T cell lines and clones. *Scand. J. Immunol.* **22,** 295.

KLARESKOG, L., JOHNELL, O., HULTH, A., HOLMDAHL, R. & RUBIN, K. (1986) Reactivity of monoclonal anti-type II collagen antibodies with cartilage and synovial tissue in rheumatoid arthritis and osteoarthritis. *Arthritis Rheum.* **29,** 730.

LOGTENBERG, T., KROON, A., GMELIG-MEYLING, F.H.J. & BALLIEUX, R.E. (1986) Production of anti-thyroglobulin antibody by blood lymphocytes from patients with autoimmune thyroiditis, induced by the insolubilized autoantigen. *J. Immunol.* **136,** 1236.

MORGAN, K., CLAGUE, R.B., COLLINS, I., AYAD, S., PHINN, S.D. & HOLT, P.J.L. (1989) A longitudinal study of anti-collagen antibodies in patients with rheumatoid arthritis. *Arthritis Rheum.* **32,** 139.

STUART, J.M., TOWNES, A.S. & KANG, A.H. (1981) Nature and specificity of the immune response to collagen in type II collagen-induced arthritis. *J. clin. Invest.* **69,** 673.

TARKOWSKI, A., KLARESKOG, L., CARLSTEN, H., HERBERTS, P. & KOOPMAN, W.J. (1989) Secretion of antibodies to types I and II collagen by synovial tissue cells in patients with rheumatoid arthritis. *Arthritis Rheum.* **32,** 1087.

'T HART, L.A., SIMONS, J.M., KNAAN-SHANZER, S. & LABADIE, R.P. (1990) Anti-arthritic activity of the newly developed neutrophil oxidative burst antagonist apocynin. *Free Radical Biol. Med.* (in press).

TRENTHAM, D.E. (1982) Collagen arthritis as a relevant model for rheumatoid arthritis. Evidence pro and con. *Arthritis Rheum.* **25,** 911.