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Origin of the autoreactive anti-type II collagen response

I. FREQUENCY OF SPECIFIC AND MULTISPECIFIC B CELLS IN PRIMED MURINE LYMPH NODES

R. HOLMDAHL*, M. ANDERSSON* & A. TARKOWSKI† * *Department of Medical and Physiological Chemistry, Uppsala University, Uppsala, and † Department of Medical Microbiology and Rheumatology, University of Göteborg, Göteborg, Sweden*

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SUMMARY

DBA/1 mice develop systemic polyarthritis and high titres of collagen autoantibodies after immunization with type II collagen. In this study we have analysed the frequency and specificity of antibody-producing B cells in draining lymph nodes 11 days after a primary immunization with rat type II collagen. A remarkably high frequency of type II collagen-reactive hybridomas (30%, $n=71$), the majority cross-reacting with autologous type II collagen, were generated. Most of the collagen-reactive hybridomas produced IgG antibodies specific for type II collagen. Many other hybridomas produced multispecific antibodies (9%): these were preferentially of the IgM class. Some of the monoclonal multispecific hybridomas produced antibodies that cross-reacted with specific epitopes on the collagen molecule. One type II collagen-reactive hybridoma also displayed rheumatoid factor activity. Different mechanisms for the generation of the high frequency of collagen reactive antibodies are discussed.

INTRODUCTION

Collagen-induced arthritis in rodents is an experimental autoimmune model that provides the opportunity to investigate the role of a defined autoantigen: cartilage-derived type II collagen. Both T-cell and B-cell mediated immune responses have been implicated as having important roles in the pathogenesis of collagen-induced arthritis (Stuart & Dixon, 1983; Holmdahl *et al.*, 1985b). It has been shown that both B cells and T cells from arthritis-susceptible strains can be activated with autologous type II collagen (Holmdahl *et al.*, 1985a, b). Furthermore, it is possible to induce arthritis after immunization with autologous type II collagen (Trentham, Townes & Kang, 1977; Holmdahl *et al.*, 1985a). The induction of collagen arthritis, as well as the induction of an antibody response towards type II collagen, are intimately associated with expression of certain class II MHC genes (Wooley *et al.*, 1981; Holmdahl *et al.*, 1986a). Recently, it has been proposed that collagen immunization also results in the generation of rheumatoid factors (Wolf *et al.*, 1986, Holmdahl *et al.*, 1986c, 1987). We have described monoclonal rheumatoid factors isolated from collagen-immunized and arthritic DBA/1 mice: one type of rheumatoid factor cross-reacting with type II collagen and another type cross-reacting with a common idiotope on anti-type II collagen antibodies (Holmdahl *et al.*, 1986c, 1987). These findings suggest that auto-antibodies related to type II collagen may play an important role in immune regulation and, in this context, may be an example of dysregulation in the control of pathogenic autoimmunity.

This paper is the first in a series aimed at characterizing the origin and specificities of the murine immune response to type II collagen. Here is reported that a high frequency of anti-type II collagen B cells is activated in draining lymph nodes 11 days after a primary immunization with rat type II collagen. All hybridomas from the fusion are characterized according to specific contra-multispecific interactions with various antigens. autoreactivities and isotypes.

Abbreviations: DNP, dinnitrophenylphosphate; ELISA, enzyme-linked immunosorbent assay; Ig, immunoglobulin; PBS, phosphate-buffered saline.

Correspondence: Dr R. Holmdahl, Dept. of Medical and Physiological Chemistry, Box 575, 751 23 Uppsala, Sweden.

MATERIALS AND METHODS

Animals

DBA/1 and B10P mice originally obtained from Jackson Laboratory (Bar Harbor, ME) and B10G mice originally obtained from Olac (Bicester, Oxon, U.K.) were kept and bred at the Biomedical Center, Uppsala, and used at 8 weeks of age.

Antigens

Pepsin-modified cartilage collagens were prepared from mouse

processus xiphoideus and rat chondrosarcoma using essentially the same methodology as described previously (Miller, 1972). Denaturation of rat type II collagen was performed at 50° for 30 min immediately prior to use. Rat skin type I collagen was prepared as described previously (Chung & Miller, 1974). Normal rabbit IgG was purified with protein A absorption techniques. Purification and purity of the normal DBA/1 IgG Fc fragments are described elsewhere (Holmdahl *et al.*, 1986c). Chick ovalbumin (grade V) and globulin-free bovine serum albumin (A0281) were purchased from Sigma (St Louis, MO) and tuberculin (PPD) from Statens Seruminstitut, Copenhagen, Denmark. F-actin, double-stranded DNA and cardiolipin were generously provided by Dr Renee Norberg at Statens Bakteriologiska Laboratorium, Solna, Sweden.

Hybridoma production

The immunization protocol has been previously described (Holmdahl, Moran & Andersson, 1985c). The collagen was used at 2 mg/ml in 0·1 M acetic acid and emulsified with an equal volume of complete Freund's adjuvant (Difco, Detroit, MI) using a Sorvall homogenizator. Fifty microlitres of this emulsion containing 50 μg of type II collagen were injected subcutaneously into the hind footpads of male mice. After 11 days the mice were killed and the draining lymph nodes (popliteal and inguinal) removed and used as a source for antibody-producing cells.

The myeloma cell line X63-653-Ag8 was used as a fusion partner. All cultures were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with glutamine (10 mM), penicillin (100 U/ml), streptomycin (100 μg/ml), 2-mercaptoethanol (5×10^{-5} M) and 10% fetal calf serum (KC Biological, Lenexa, KS) (complete medium). Fusion and the establishment of antibody-producing hybridomas were essentially performed as described by Köhler & Milstein (1975). Briefly, the lymph nodes were gently dispersed and the resulting cell suspension freed from debris by sedimentation and washed once in PBS. The primed lymph node cells (10^8) were subsequently mixed with the myeloma cells (10^7) and fused in 50% (w/v) polyethylene-glycol (MW 2000). The suspended cells were then distributed in five flat-bottomed microtitre plates (Nunc, Roskilde, Denmark) in a volume of 100 μl/well in complete medium together with 5×10^6 fresh rat thymocytes/ml. The plates were incubated at 37° in 7·5% CO_2 in air. After 24 hr the cultures were supplemented with optimal concentrations of hypoxanthine, thymidine and aminopterine in complete medium (100 μl/well). The plates were screened macroscopically for growing clones. The clones were transferred to 24-well plates with 16 mm wells (Costar, Cambridge, MA) when the largest clones covered approximately one-third of the bottom in the microtitre plate wells. The clones were grown in the larger wells until the largest clones covered approximately one-third of the bottom of the plate. Then, the supernatants were collected and stored at −20° until used. Selected hybridomas were subcloned by limited dilution technique at a concentration of 0·5 cell/well.

Antibody purification

Hybridomas were cultured in complete medium for 2 weeks. Then the culture supernatant was harvested by centrifugation (20,000 *g*). Different IgG subclasses were purified on protein A–Sepharose columns. IgM antibodies were purified on a rabbit anti-mouse IgM–Sepharose column and eluted with 3 M $MgCl_2$. The purified antibodies were dialysed extensively against PBS, sterile-filtered, and the immunoglobulin content was determined from the absorbance at 280 nm.

Enzyme-linked immunosorbent assay (ELISA)

Micro-ELISA plates (Dynatech, Plochingen, FRG) were coated overnight at 4° with 50 μl/well of phosphate-buffered saline containing 10 μg/ml of the various antigens. All subsequent incubations were made in a volume of 50 μl/well. Washings were done with Tris-buffered saline (pH 7·4) containing 0·05% Tween 20 (Tris–Tween) with the help of an ELISA washing device (Skatron, Norway).

The amount of antibody bound to the different antigens was estimated after incubation with an anti-mouse kappa monoclonal antibody (187·1) (Ware, Reade & Der, 1984) coupled to alkaline phosphatase. We have found this monoclonal anti-mouse kappa antibody to be superior to polyclonal reagents because it has an equal affinity to at least 95% of all mouse monomeric immunoglobulins. The subsequent quantification of bound enzyme was performed by incubation with a paranitrophenol-containing substrate buffer. The incubation time was 60 min in all hybridoma supernatant assays, and between 60 and 90 min when the various subcloned hybridomas were tested. The absorbance values were determined in a Titertek Multiscan spectrophotometer.

Competition ELISA

For analysis of the reactivity of the different monoclonal antibodies to different epitopes on the native type II collagen molecule, we used an avidin–biotin based system as previously described (Holmdahl *et al.*, 1986d). The concentration of each biotinylated monoclonal antibody that gave 75% of the maximal absorbance value was predetermined. Subsequently each biotinylated antibody at this submaximal concentration was allowed to compete with 50 μg/ml of each unlabelled monoclonal antibody for binding to rat type II collagen. The degree of binding of biotinylated antibody was quantified with an avidin–alkaline phosphatase conjugate.

Isotype determinations

The isotypes of the antibodies in culture supernatants were analysed by means of an immunodiffusion technique using goat anti-mouse IgM, anti-IgG and IgA-specific antibodies (Meloy, Springfield, VA). The isotypes of the purified monoclonal antibodies were determined in ELISA with horseradish peroxidase-conjugated mouse isotype-specific goat antibodies (Nordic Laboratories, Tilburg, The Netherlands).

RESULTS

Native rat type II collagen, emulsified in complete Freund's adjuvant, was used for immunization of DBA/1 male mice in the hind footpads. Other DBA/1 male mice were immunized with denatured rat type II collagen, which is poorly immunogenic. Also, mice with the B10 background were immunized with native rat type II collagen: B10P mice, which are low responders and B10G, which are high responders for a collagen antibody response (Holmdahl *et al.*, 1986a). Eleven days after immunization the draining lymph nodes were used as a source of B-cell blasts, hybridized and distributed in microtitre plates. The results show that immunization of high responder mice (DBA/1

Table 1. Results of fusions

Immunogen	Mouse strain	Number of seeded wells	Number of wells with growing cells	Estimated monoclonality*	Number of hybridomas	
					Reactive with collagen II†	Specific for collagen II‡
Native type II collagen	DBA/1	480	240	70%	72 (30%)	67 (28%)
Denatured type II collagen	DBA/1	1440	490	81%	29 (6%)	15 (3%)
Native type II collagen	B10P	960	193	88%	9 (5%)	8 (4%)
Native type II collagen	B10G	960	65	95%	40 (63%)	40 (63%)

* Estimated frequency of wells containing only one clone of hybridoma (De Blas, Ratnaparkhi & Mosimann, 1981).

† Binding to the immunogen in ELISA. Absorbances above 0·100 are calculated as positive.

‡ All supernatants also determined for binding to ovalbumin and PPD in ELISA. Specific hybridomas had no cross-reactive binding to these antigens.

Table 2. Specificities and isotypes of antibodies generated in the fusion experiment with native rat type II collagen-primed lymph node cells

	Hybridomas reactive with:					
	Native rat Type II Collagen	Native mouse Type II Collagen*	Ovalbumin	Rabbit IgG	Tuberculin (PPD)	All hybridomas
Number of hybridomas:	71	47	4	12	12	240
% IgG	68	68	25	42	18	50
% IgM	20	23	75	42	36	25
% IgG + IgM†	6	6	0	8	27	10
% IgA	4	2	0	8	9	3
% Ig?‡	3	0	0	0	9	3
% No Ig§						8

* The vast majority was specific for type II collagen and cross-reacted with rat type II collagen.

† Reactive with both IgG and IgM, indicating occurrence of antibodies with both isotypes.

‡ Uncertain determination of isotype in the immunodiffusion analysis.

§ No immunoglobulin detected in the immunodiffusion analysis.

and B10G) with native rat type II collagen induced a high frequency (30–63%) of collagen-specific hybridomas (Table 1).

Supernatants from all wells containing growing hybridomas, derived from DBA/1 mice immunized with native rat type II collagen, were analysed for their reactivity with a number of different antigens (Table 2 and Fig. 1). A large fraction of the hybridomas (30% of the wells) obtained from DBA/1 mice immunized with native rat type II collagen produced antibodies reactive with native rat type II collagen, the majority detecting determinants shared between rat and mouse collagen. Most of these immunogen-specific antibodies were of the IgG class. A minor fraction (9%) of the hybridomas produced antibodies reactive with other antigens such as ovalbumin (2%), rabbit IgG (5%) and PPD (5%). The vast majority of the hybridomas

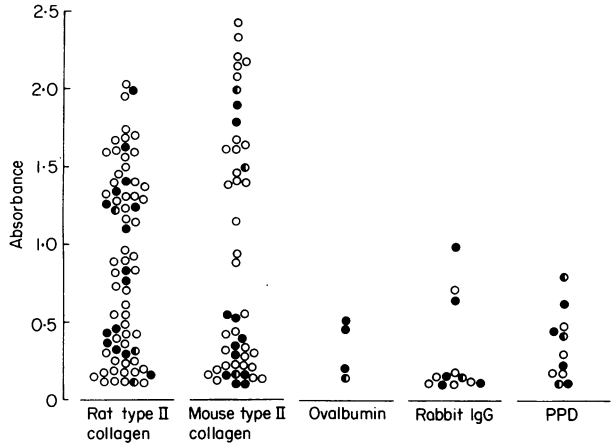

Figure 1. Specific reactivities of the various hybridomas generated after fusion of primed lymph node cells. Absorbance values exceeding 0·100 OD in an ELISA are indicated in the figure. Filled symbols represent IgM, half filled are both IgM and IgG, and open symbols are non-IgMs, preferentially IgG.

displaying reactivities with these presumably irrelevant antigens produced antibodies that were cross-reactive, of the IgM class, and that produced weak absorbance values in the ELISA.

A number of both collagen-specific and multispecific hybridomas from the fusion were subcloned and the respective monoclonal antibodies purified. The antigen specificities of these antibodies are shown in Table 3. All hybridomas displayed similar reactivities as in the primary screening. Thus, three antibodies were of the immunogen-specific type (F4, F9 and F10), one cross-reacted with IgG (F6), one had an unknown specificity (F3), and three were of the multispecific type (F2, F5 and F8). These latter antibodies bound to a large variety of different antigens including double-stranded DNA, F-actin and autologous IgG Fc fragments. Virtually each antibody displayed a different pattern of reactivities indicating that the binding was determined by the variable regions of the antibody molecule. For example, the F2 antibody bound to double-stranded DNA, F-actin and type II collagen, F8 bound to BSA and PPD, and F5 bound to F-actin and type II collagen.

In addition, antibodies with a high reactivity for type II collagen were analysed for binding to specific epitopes on the collagen molecule. We have previously identified six different epitopes by a biotin–avidin based competition ELISA method. These epitopes could be defined by certain specific monoclonal antibodies (C1, C2, E8, B1 and D3μ monoclonal antibodies). As has previously been discussed (Holmdahl *et al.*, 1986d), the epitopes defined by the different monoclonals could be either structurally identical or juxtaposed because of steric hindrance. We have now included the presently characterized monoclonal antibodies in the same system (Table 4). The two analysed immunogen-specific antibodies (F4 and F10) bound to specific epitopes: the D3μ defined epitope (F4) and the E8 defined epitope (F10). Also, the multispecific antibodies were epitope-specific since both the F2 and F5 antibodies bound to the D3μ defined epitope.

DISCUSSION

It is described here that lymph nodes from the collagen-induced arthritis-susceptible strain DBA/1 contain a large fraction of B cells producing autoreactive anti-type II collagen antibodies as early as 11 days after immunization with rat type II collagen.

It has earlier been shown that the generation of anti-type II collagen antibodies is a T-cell dependent process (Klareskog *et al.*, 1983). We have found that immunization with rat type II collagen gives rise to both T cells that do not cross-react and T-cells that do cross-react with mouse type II collagen (Holmdahl *et al.*, 1985a, 1986b). We do not know whether both these types of T cells can help B cells in antibody production. This is,

Table 3. Cross-reactive properties of subcloned and purified monoclonal antibodies originating from the fusion of lymph node cells primed with rat type II collagen

Monoclonal antibody	Isotype	Antigens*										
		OVA	PSA	PPD	F-actin	dsDNA	Cardiolipin	IgGFC	IgGFab	B1Fab	Native rat CI	Native rat CII
F2	IgM	0	0·83	0·36	1·05	1·54	0·03	0·37	0·13	1·50	0·03	1·30
F3	IgG2a	0	0	0	0	0	0	0	0	0	0	0
F4	IgG2a	0	0	0	0	0	0	0	0	0	0	>2·0
F5	IgM	0	0·09	0·10	0	0·36	0·04	0	0·05	0·15	0	>2·0
F6	IgG2b	0	0	0	0	0	0	0·38	0	0	0	0·50
F8	IgM	0	1·56	0·50	1·05	0·21	0·06	0·13	0·13	1·35	0·01	0·55
F9	IgM	0	0	0	0	0·05	0	0	0	0	0	0·37
F10	IgG1	0	0	0	0	0·01	0	0	0	0	0	0·37

* The results are expressed as the absorbance values at an antibody concentration of 1 μg/ml in ELISA. The following antigens were used at a coating concentration of 10 μg/ml: ovalbumin (OVA), bovine serum albumin (BSA), purified protein derivate (PPD), F-actin, double-stranded DNA (dsDNA], cardiolipin, normal DBA/1 IgG Fc fragments (IgGFc), normal DBA/1 IgG Fab fragments (IgGFab), Fab fragments purified from a type II collagen-reactive monoclonal lambda antibody derived from DBA/1 (B1Fab), native rat type I collagen (native rat CI) and native rat type II collagen (native rat CII). The absorbance values obtained after binding to IgGFab are not fully comparable to the values obtained with the other antigens since a different conjugate, a goat anti-mouse μ-chain linked with alkaline phosphatase, was used. All other assays were performed with an alkaline phosphatase-labelled anti-mouse kappa conjugate as the secondary reagent.

Table 4. Reactivities with different epitopes on type II collagen of antibodies derived from the NRC fusion

Labelled monoclonal antibodies: (μg/ml)	Unlabelled monoclonal antibodies (50 μg/ml								
	C1	C2	E8	F10	B1	D3u	F2	F4	F5
C1 (0·150)	96	0	0	0	0	0	0	0	0
C2 (0·025)	0	92	46	33	0	0	0	0	0
E8 (0·200)	0	0	100	97	0	0	0	0	28
F10 (0·850)	0	0	89	82	0	0	0	0	0
B1 (0·350)	0	0	0	0	91	0	0	0	0
D3μ (0·075)	0	0	0	0	0	100	56	86	61
F2 (1·000)	0	0	0	0	0	77	68	84	33
F4 (0·030)	0	0	0	0	0	100	72	100	33
F5* (1·000)	0	0	0	0	0	100	0	0	100

Percentage inhibition of biotinylated monoclonal antibodies (in submaximal concentrations) by unlabelled monoclonal antibodies (50 μg/ml) for binding to native rat type II collagen.

* Competition assays with the F5 antibody were performed with native mouse type II collagen as the antigen.

however, probable since anti-type II collagen antibody production can be induced with both heterologous and autologous type II collagen with higher amounts of antibodies produced after immunization with heterologous type II collagen (Holmdahl *et al.*, 1985a). The presence of T-helper cells reactive with autologous type II collagen could possibly explain the observed high frequency of type II collagen-reactive B cells. If so, many of these B cells may be memory cells. It has been shown that the majority of the antibodies generated 7–14 days after primary immunization with the T-cell dependent antigen DNP-haemocyanin was produced by memory B cells (MacLennan & Gray, 1986).

Another approach to explain the high frequency of autoreactive B cells in the present system is to postulate the occurrence of collagen-reactive B cells in the natural repertoire. In fact, it has been suggested that the natural B-cell repertoire is self-centred, or in other words continuously stimulated by self antigens (Jerne, 1955; Coutinho *et al.*, 1984). It has been demonstrated that B cells comprising the natural repertoire react with a large variety of different self antigens (Dighiero *et al.*, 1983; Holmberg *et al.*, 1984). Most of these auto-reactive 'natural' B cells produce multispecific IgM antibodies and are probably activated independently from specific T cells. Also among the present collection of hybridomas are certain clones (for example F2, F5 and F8) that secrete antibodies with the same characteristics as natural antibodies. They are multispecific, preferentially of the IgM class, and probably react with low affinity to the different antigens as judged from ELISA titration curves (data not shown). Notably, some of the analysed multispecific antibodies display commonly described cross-reactivities such as between DNA and IgG-Fc. Furthermore, some of the multispecific hybridomas cross-react with type II collagen and bind to certain epitopes on the collagen molecule, implicating a specific interaction.

Another possibility for the generation of collagen autoantibodies is provided by the recently described connection between rheumatoid factor production and collagen immunity (Holmdahl *et al.*, 1986c, 1987; Wolf *et al.*, 1986) and the finding that rheumatoid factors are common in the natural antibody repertoire (Shlomchik *et al.*, 1986). Therefore, the hybridomas were also screened for reactivities against rabbit IgG. We found that most polyspecific hybridomas reacted with the rabbit IgG used in the primary screening of hybridoma supernatants. The reactivities with IgG were also sustained after subcloning and purification of the antibodies. However, the multispecific IgM antibodies were not specific for the Fc part of IgG. On the other hand, we found one hybridoma (F6) that produced IgG2b antibodies that specifically reacted with both syngeneic IgG Fc and type II collagen. These properties are similar to another monoclonal rheumatoid factor generated from the DBA/1 mouse that we have previously described (Holmdahl, 1986c, 1987).

Although different mechanisms for the generation of the high frequency of anti-type II collagen autoantibodies can be postulated, the different suggestions are not exclusive of each other. The autoimmunity generated against type II collagen, expressed by both T cells and B cells, and resulting in erosive arthritis, provides a model for investigating the different mechanisms for the origin of pathogenic autoimmunity.

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