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Contribution of the spleen, lymph nodes and bone marrow to the antibody response in collagen-induced arthritis in the rat

J. RAHMAN & N. A. STAINES *Immunology Section, King's College, London, England*

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SUMMARY

The relative contribution of different lymphoid tissues to the anti-CII antibody response was studied in rats with arthritis produced as a result of immunizing them with collagen type II (CII). Antibody production was measured by maintaining lymphoid cells in short-term culture in collagen-coated microculture wells: the antibody they secreted was determined directly by a modified ELISA. Systemic sensitization to CII was established within a week of immunization, and a stronger response in the local draining lymph nodes relative to the spleen was associated with the development of clinical disease. From experiments involving splenectomy and adoptive cell transfer, the spleen was ascribed a suppressive role in controlling both arthritis and total antibody production. The bone marrow was found to be an important site of antibody production and the greater production of antibody by cells from tibial marrow in limbs with arthritis, compared with healthy limbs, argues for a local immune response to degrading joint antigens that may have systemic suppressive or protective properties. It is concluded that local immunity reflects the state of disease and that the antibodies produced by different lymphoid tissues may be made in response to different stimuli, and that the antibodies in turn may have different pathological effects.

Keywords collagen arthritis anti-collagen-antibodies bone marrow spleen suppression

INTRODUCTION

The immunization of rats with soluble native type II collagen (CII) may induce a polyarthritic disease. There is an accompanying immune response to CII involving the activation of both T cells and B cells and this activity implies that the disease is autoimmune in origin. This is reinforced by several observations: lymphoid cells from immune rats can adoptively transfer disease itself, or resistance to disease induction (Trentham, Dynesius & David, 1978; Burrai *et al.*, 1985); athymic rats are refractory to induced disease (Klareskog *et al.*, 1983); and passively transferred antibody can cause transient inflammatory joint lesions (Stuart & Dixon, 1983) or prevent subsequent induction of disease (Staines *et al.*, 1981; Englert *et al.*, 1985). Although immunization with CII over a wide-dose range and by several different parenteral routes will readily induce specific immune responses to CII there are, however, stringent requirements for inducing arthritis. In rats, the CII must be emulsified in Freund's incomplete adjuvant (FIA) and be injected intradermally; below a dose of 100 μg of CII, arthritis never appears in rats of the strain we normally use and as the dose is increased, the proportion of animals developing frank clinical disease increases to a maximum of 98%.

Whereas anti-CII serum antibody titres tend to relate to the incidence of disease in groups of experimental animals, it is the case that appreciable and comparable titres of IgG class anti-collagen antibodies can be found in both arthritic and non-arthritic members of a group. However, there may be quantitative differences in the amounts and relative functional affinity of anti-CII antibodies of particular IgG subclasses produced in non-arthritic and arthritic rats (Firth *et al.*, 1984; Thompson *et al.*, 1988) and mice (Kresina & Finegan, 1986; Nagler-Anderson *et al.*, 1986; Staines *et al.*, 1990). The reasons why all animals that have been immunized identically fail to respond similarly are not known. It might be anticipated that activation or systemic sensitization of distant lymphoid tissues after local intradermal immunization would occur in characteristic patterns. Different lymphoid tissues might be expected to produce antibodies qualitatively and quantitatively different from each other. For these reasons we have studied the anatomical distribution of antibody production in rats immunized with CII and report here different patterns of activity characteristic of arthritic and non-arthritic rats.

J.R. present address: Department of Neuropathology, Institute of Psychiatry, Denmark Hill, London, UK.

Correspondence: Professor Norman A. Staines, Immunology Section, King's College London, Campden Hill Road, London W8 7AH, UK.

MATERIALS AND METHODS

Animals

Inbred 3–4-month-old female WA/KIR rats were obtained

from the Kennedy Institute, London, UK, and were maintained in solid-bottomed plastic cages with wood shavings as litter. Rats were maintained on rat diet 86 (Grain Harvesters, Kent, UK) and water *ad libitum*.

Induction and assessment of arthritis

Collagen from bovine nasal septa was enzymatically solubilized, purified, dissolved in ethanoic acid and injected intradermally in 0·25 ml emulsion (2:3 antigen: FIA) in the suprascapular region site as described previously (Staines *et al.*, 1981). Controls consisted of normal sex- and age-matched rats, some of which were injected with FIA. Rats were assigned to experimental groups at random and all animals within each experiment received CII from the same batch. Animals were weighed regularly and assessed for disease. Severity of arthritis in each paw was scored on a scale of 0–4 based on increasing periarticular erythema and swelling of soft and hard tissues, and an arthritic index calculated as described elsewhere (Burrai *et al.*, 1985).

Splenectomy

Splenectomy was performed using ventral laparatomy. Anaesthesia was maintained by hypnorm (Jenssen, Beerse, Belgium) (50 μl/100 g body weight, intramuscularly) and valium (Roche Products, Herts, UK) (50 μl/100 g body weight, intraperitoneally). The splenic vessels were ligated prior to removal of the spleen and the abdomen closed with steel clips. Sham splenectomy was performed in an identical fashion, including mobilization but not ligation or removal of the spleen. Animals were immunized 6 days after surgery. The completeness of splenectomy and absence of splenosis were confirmed at autopsy.

Estimation of serum anti-CII antibody levels

Blood samples were obtained from the ventral tail artery and sera were stored at −20°C. Anti-CII antibody levels were determined using an ELISA (Thompson & Staines, 1986) with a blocking step (100 μl/well of PBS containing 2% casein) (BDH, Poole, UK) for 30 min at 37°C. The results are expressed as midpoint serum titres ($\log_{10}$).

Cell suspensions

Rats were killed at times indicated in the text and their tissues removed. Bone marrow cells were collected by flushing each tibia with 10 ml handling medium (Dulbecco's PBS containing calcium and magnesium, 2% heat-inactivated (56°C, 30 min) fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin) (GIBCO, Paisley, UK) and the cells dispersed by passing them repeatedly through a 23-gauge hypodermic needle. Other tissues were teased gently with fine forceps to release the cells and all suspensions were washed twice (1500 *g* for 10 min at 4°C) and finally resuspended in RPMI 1640 culture medium containing 12·5 mM HEPES, 5% heat-inactivated fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin. Cell suspensions were maintained on melting ice at all times and their numbers and viability assessed by gentian violet uptake and trypan blue exclusion.

Antibody production

Antibody production by cells of the different tissues was assessed by modification of published methods (Kelly, Levy & Sikora, 1979; Weetman, McGregor & Hall, 1982). In these experiments, CII diluted to 10 μg/ml in PBS was filter sterilized (0·45-μm filter; Millipore, Harrow, UK) and 50 μl/well used to coat 96-well flat-bottomed tissue culture plates (Nunc, Roskilde, Denmark) for 1 h at 37°C. Wells were washed three times with sterile PBS and sites of uncoated plastic were blocked with 100 μl/well 1% sterile BSA (30 min at 37°C). Wells were washed three times with sterile PBS and cell suspensions at various doubling dilutions were dispensed in 100-μl aliquots (four replicates/dilution) into them. Each well was made up to 200 μl with medium. Wells containing medium alone or lymphocytes from FIA-injected or normal rats served as controls. Tripling dilutions of a standard pooled anti-CII antiserum (three replicates/dilution) were included in each plate to serve as a standard for the estimation of anti-CII antibody. Plates were incubated for 18 h at 37°C in 5% in CO_2 in air and 100% relative humidity. Following culture, cells were removed with four washes of PBS containing 0·1% Tween 20 (Sigma Chemical Co., Poole, UK) and bound antibody estimated by the standard ELISA protocol except that development with OPD was for 60 min. The concentration of collagen and the incubation times quoted were established experimentally to give the highest sensitivity possible in the assay.

Calculation of results

Optical density (OD) values corresponding to the antibody produced by cells in culture were used to construct antibody titration profiles, and a representative example of this is shown in Fig. 1a. The interpolated ELISA OD reading given by 10^6 viable nucleated cells was referred to the standard antibody titration curve (Fig. 1b) and the corresponding (interpolated) serum dilution read from it; this value was multiplied by 10^5 and the result defined as the units of antibody produced by 10^6 cells. Results are generally presented in derived form as the total amount of antibody produced in each tissue to illustrate the contribution that each makes to the total antibody response. There were no significant changes in the cellularity of the individual tissues during the time of the experiments described

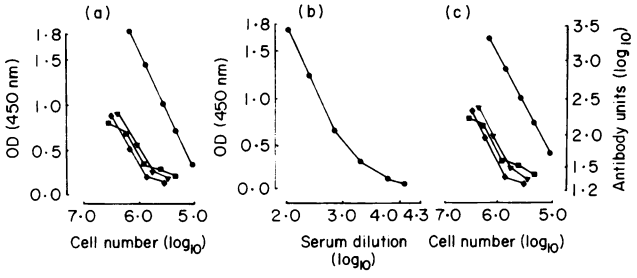

Fig. 1. Representative titration curves for antibody secreted in microculture cell ELISA and the relationship between the number of cells in culture, optical density (OD) and units of antibody. Cultures were set up with cells from the brachial lymph nodes (●), spleen (▼), right tibial bone marrow (■) and left tibial bone marrow (◆) from a rat 22 days after immunization with 300 μg CII in FIA. OD values corresponding to the antibody produced by cultures containing different number of cells are shown in (a). These were referred to the standard antibody titration curve (b) and the amounts of antibody made in each culture calculated are shown in (c). The transformation of OD values to Units of antibody ($\log_{10}$) illustrates the direct relationship between the number of cells in culture and the amounts of antibody they produce.

here and similar results were obtained when antibody production was calculated/10^6 cells. Bone marrow data are given in this form because of the difficulty in achieving a quantitative recovery of cells. As illustrated, the assay was consistently linear at 10^6 cells/well, and for this reason results were calculated at this point. The direct relationship between ELISA OD readings and antibody produced by cells in culture is illustrated by the transformation of OD values to units of antibody (Fig. 1c).

Cell transfer

Donor rats were injected with either 600 μg CII in FIA or ethanoic acid in FIA. Animals were killed 8 days later and their spleens and lymph nodes removed asceptically. Tissues were teased with fine forceps and the released cells suspended and washed three times (1500 *g* for 10 min) in RPMI 1640 medium containing 10% heat-inactivated fetal calf serum. Recipient rats (the same age and sex as the donors) were injected with 10^8 nucleated pooled brachial lymph node or spleen cells from the CII-immunized or control donors via the caudal vein. All recipients and uninjected controls were challenged immediately with 600 μg CII in FIA. Eight days after the initial cell transfer and challenge with CII, the original recipient rats were again injected with nucleated pooled brachial lymph node or spleen cells from CII immunized or control donors.

Recipient animals were challenged with 500 μg of soluble CII administered intraperitoneally in 1 ml of 0·1 M ethanoic acid 21 days after the initial cell transfer.

Statistical analysis

Continuous variables were analysed by their group means (Student's two-tailed *t*-test) and discontinuous variables by the Mann–Whitney *U*-test. Linear regression was determined using Pearson's product moment correlation coefficient and parallel linearity tested for group samples by comparing variance of column means about the regression line with the variance within the columns.

RESULTS

The development and pattern of anti-collagen antibody production in different local lymphoid tissues

Eight days after rats had been intradermally immunized, anti-CII antibody was produced not only by cells from their brachial lymph nodes draining the injection site but also by cells from their bone marrow and spleen. Both in terms of the amount of antibody produced per cell and the total produced by individual tissues, the brachial nodes made the most and bone marrow the least (Fig. 2a). At this time, clinical symptoms of disease were not seen, antibody production by popliteal lymph node cells was not detected and serum antibodies were present in low titre. After 3 weeks, however, 64% of the rats had become arthritic, and in both arthritic and non-arthritic rats, antibody was produced in the same tissues but at higher levels than at day 8 (Fig. 2b). The same hierarchy of response was seen and antibody was produced, in relatively small amounts, by cells from the popliteal lymph nodes (data not shown).

Comparing the arthritic and non-arthritic animals, they produced, on average, similar amounts of antibody in the spleen and bone marrow. The response of the brachial node cells in terms of total levels of antibody production was significantly higher in arthritic compared with non-arthritic rats. Although

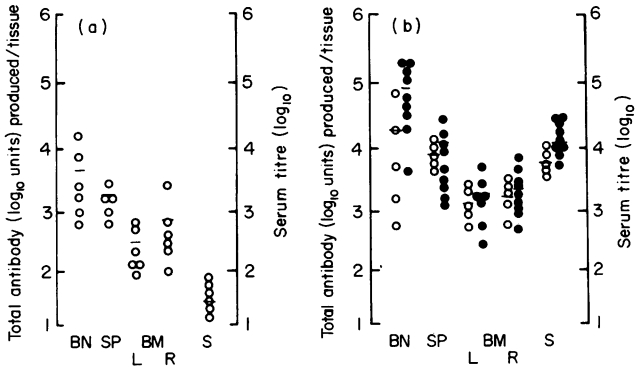

Fig. 2. Anti-CII antibody produced by cells from pooled brachial lymph nodes (BN), spleen (SP), left (L) and right (R) tibial bone marrow (BM) and serum antibody titres (S) in rats immunized with 300 μg CII in FIA, 8 days (a) and 22 days (b) after immunization. Open symbols, non-arthritic rats; closed symbols, ● arthritic animals. Horizontal bars correspond to geometric mean values. Cells from brachial lymph nodes from arthritic rats produce significantly higher levels of antibody compared with those from non-arthritic rats ($P < 0{\cdot}05$).

the response of the popliteal lymph node cells was higher in arthritic rats, it was not significantly different. More antibody was produced in the ipsilateral than in the contralateral marrow of animals with unilateral hind-limb arthritis when antibody production was compared on a cell-to-cell basis (Table 1). Further, in the non-arthritic contralateral limbs of these animals, less antibody was made than in the bone marrow of immunized animals that did not present with clinical disease in either limb, suggesting that the presence of arthritis in one limb may lead to a reduction in antibody production in distant tissues.

Local antibody production and its relation to developing clinical disease

The ratios of the amounts of antibody produced by the brachial node cells to those produced by the spleen cells were different in non-arthritic rats at 8 or 22 days compared with arthritic rats at the later time. No other comparisons of paired tissues showed such differences. This relationship between antibody produced in the brachial nodes and spleens of individual animals can be seen clearly in a regression analysis (Fig. 3). In the pre-clinical phase there was an inverse relationship between antibody production by the two tissues and although antibody production by the spleen increased, this relationship was maintained in animals that had not developed disease by 3 weeks: the regression lines are parallel. In those animals that did develop arthritis, however, the relationship changed to reveal a significant positive association between the activity in the two tissues. These data imply that the balance between the levels of antibody production from the lymph nodes and the spleen is critical in the induction and the suppression of disease and that a certain threshold of antibody production develops in the nodes draining the injection site before the development of arthritis and a (high) antibody response in the spleen.

Effects of splenectomy on antibody production and arthritis

Because the previous experiment suggested a role for the spleen in the development of disease, rats were splenectomized 6 days before intradermal immunization with CII. Splenectomy led to

Table 1. Anti-CII antibody production by cells in the tibial bone marrow and its relation to arthritis in adjacent feet

Anti-CII antibody produced ($U/10^6$ cells)					
Arthritic rats			Non-arthritic rats (All limbs)	All rats	
Arthritic limbs ($n=12$)	Non-arthritic limbs* ($n=5$)	All limbs ($n=17$)	($n=10$)	Arthritic limbs ($n=12$)	Non-arthritic limbs ($n=15$)
$50{\cdot}1 \pm 30{\cdot}0$	$18{\cdot}2 \pm 8{\cdot}2$	$40{\cdot}8 \pm 29{\cdot}0$	$38{\cdot}0 \pm 13{\cdot}0$	$50{\cdot}1 \pm 13{\cdot}0$	$31{\cdot}4 \pm 15{\cdot}1$
$P<0{\cdot}05$		$P>0{\cdot}8$		$P<0{\cdot}05$	
		$P<0{\cdot}01$			

Antibody production determined in cell ELISA system using cells from rats immunized with 300 μg CII in FIA. Values shown are means ± 1 s.d. *P* values determined by two-tailed Student's *t*-test.

* One sample was lost.

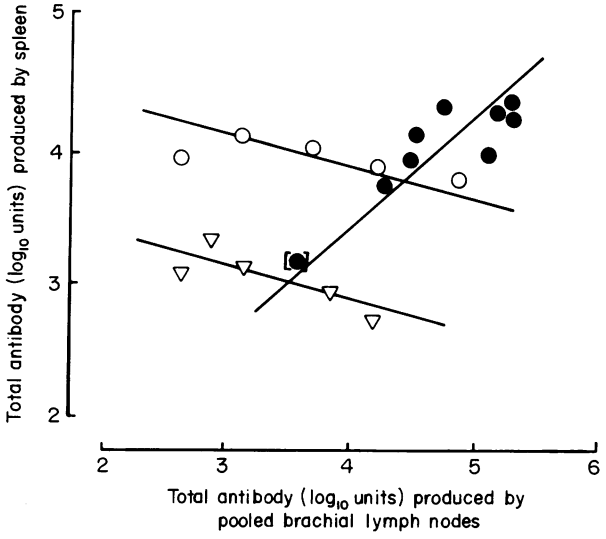

Fig. 3. The relationship between total amounts of antibody produced by cells from the spleen and brachial lymph nodes of rats immunized with 300 μg CII in FIA. Computed regression lines are fitted to data for non-arthritic animals 8 days after immunization ($r=-0{\cdot}74$, $P<0{\cdot}1$) (▽) and arthritic ($r=0{\cdot}68$, $P<0{\cdot}05$) (●) and non-arthritic ($r=-0{\cdot}75$, $P<0{\cdot}1$) (○) rats 22 days after immunization. Regression lines are parallel at 8 and 22 days for non-arthritic rats ($P<0{\cdot}05$) and both are significantly different from that for arthritic rats at 22 days ($P<0{\cdot}0001$). Excluding this point ([●]) from regression analysis for arthritic animals at day 22 ($r=0{\cdot}59$, $P<0{\cdot}2$) the regression line still remains significantly different from the two non-arthritic lines ($P<0{\cdot}0005$).

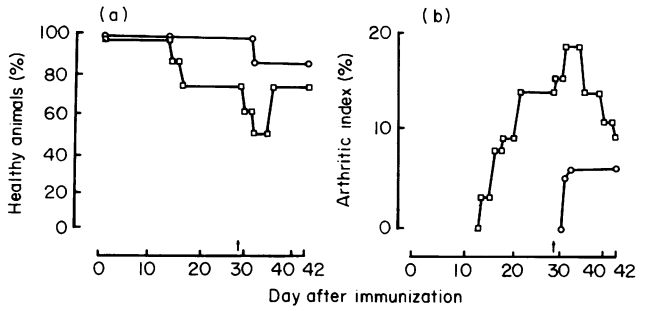

Fig. 4. Effect of splenectomy on onset and incidence of disease (a) and its severity (b). Groups of rats ($n=8$) were immunized with 250 μg CII in FIA, 6 days after splenectomy (□) or sham splenectomy (○) and boosted with 500 μg CII intraperitoneally 28 days after immunization as indicated by the vertical arrows. The arthritic index (calculated from all animals in a group) was significantly different in the splenectomized and control groups between days 12–42 inclusive (Mann–Whitney *U*-test, $P<0{\cdot}05$).

an earlier onset of disease and generally more severe arthritis (Fig. 4). Splenectomy also resulted in significantly greater antibody production in the bone marrow (total mean antibody units($\log_{10}$): splenectomized rats, 3·558; sham-operated controls, 3·217; $P<0{\cdot}05$) but not the brachial nodes (total mean antibody units ($\log_{10}$): splenectomized rats, 3·948; sham-operated controls, 3·891; $P<0{\cdot}4$). Serum antibody titres for splenectomized rats were also significantly higher at 4 weeks (splenectomized rats, 3·282; sham-operated controls, 3·010; $P<0{\cdot}04$) and at 6 weeks after immunization (splenectomized ras, 3·701, sham-operated controls, 3·366; $P<0{\cdot}02$).

Suppression of disease induction by cell transfer

Brachial lymph node cells or spleen cells were transferred to normal rats that were then immunized with CII in FIA in the normal way. Animals that received either cell type from control FIA-injected donors exhibited normal symptoms of disease starting 12 days after the cell transfer and challenge with CII in FIA. In these animals disease incidence was 100% by day 27 (Fig. 5a). In contrast, disease incidence was significantly reduced in animals that had received spleen cells from CII-immunized donors. Animals receiving brachial lymph node cells showed only a marginal change in disease incidence. Furthermore, the severity of arthritis was reduced in recipients of immune spleen cells ($P<0{\cdot}01$; Fig. 5). Serum antibody levels were not significantly affected in any recipient group (results not shown).

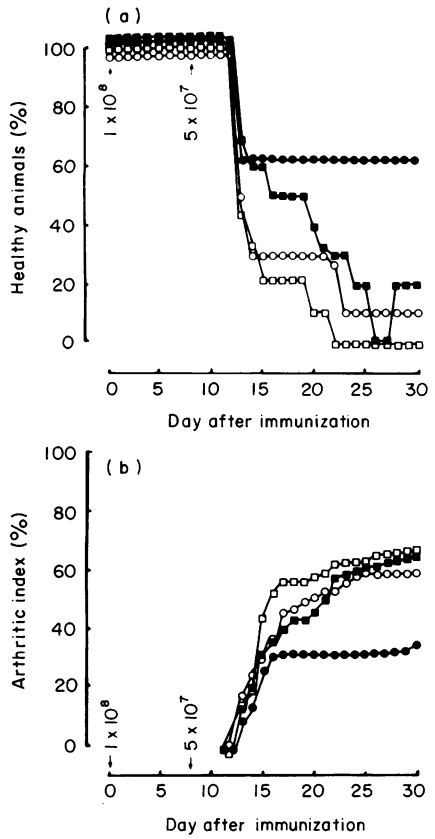

Fig. 5. Effect of adoptive transfer on onset and incidence of disease (a) and its severity (b). Recipient rats ($n=10$/group) received nucleated brachial lymph node (□) or spleen cells (■) from donor animals injected with ethanoic acid in FIA or brachial lymph node (○), or spleen cells (●) from animals immunized with 600 μg CII in FIA. The number of cells transferred are shown above the vertical arrows. All recipient animals were immunized with 600 μg CII in FIA on day 0 and boosted intraperitoneally with 500 μg CII on day 21. Donor animals were injected eight days prior to sacrifice. Spleen cells from animals immunized with CII in FIA reduced the severity and incidence of arthritis in recipient animals ($P<0{\cdot}01$).

DISCUSSION

The production of anti-CII antibodies is a conspicuous feature of collagen arthritis in rats and several studies have ascribed important roles to them in the development and regulation of the disease (Stuart *et al.*, 1980; Staines *et al.*, 1981; Watson & Townes, 1985; Holmdahl *et al.*, 1986). The present study throws new light on the relative contribution of different tissues to antibody production and the possible regulatory effects anti-collagen antibodies have on the development of arthritis.

Relatively large amounts of antibody were produced in the brachial lymph nodes that drain the site of injection of the arthritogenic stimulus early in the immune response, and as the disease progressed these nodes continued to be the major site of anti-CII antibody synthesis. It appeared that a critical level of antibody production in the brachial nodes is achieved in those animals that develop arthritis. This may be a function of the effective dose of antigen reaching the nodes and it may also be the basis for the direct relationship between serum antibody titres and disease incidence (Staines *et al.*, 1981; Firth *et al.*, 1984).

The reason that intradermal immunization is the only way of inducing collagen arthritis (with soluble antigen in rats) could be, according to these results, because it provides the means by which antigen can be delivered to distant lymphoid tissues at an appropriate rate, concentration and form. Alternatively it may facilitate the circulation and homing of T cells and/or dendritic cells (Knight *et al.*, 1983; Bos & Kapsenberg, 1986). In contrast, collagen injected intravenously never induces disease but rather it induces resistance to disease induction (Staines *et al.*, 1981; Schoen, Green & Trentham, 1982; Cremer *et al.*, 1983). We conclude that the ways the brachial lymph nodes and the spleen process CII and respond to it are different. The fact that antibody production was detected in the spleen and marrow 8 days after immunization implies that the immune system becomes systemically sensitized soon after immunization. All tissues studied contributed to antibody production. Whether this is due to transport of antigen or the migration of lymphocytes is not known, but the presence of anti-CII antibody-secreting cells in the blood noted before (Morgan, Ofosu-Appiah & Holt, 1985; Wooley *et al.*, 1989) implies that cell migration plays at least a contributory role. Since antibody production in popliteal lymph nodes never rose much above background, it appears that not all lymphoid organs become equally activated as a direct consequence of the injection of the collagen. In comparison, the brachial and axillary lymph nodes made appreciable amounts of antibody 3 weeks after immunization.

In the present experiments, arthritis developed only in the hind-limbs of the rats, and the lack of a vigorous response in the popliteal lymph nodes implies that collagen derived from degrading cartilage did not reach or did not stimulate B cells in these particular nodes. In a more general context the inflammatory response in the joints may therefore be a local (protective) mechanism that prevents systemic sensitization arising as a consequence of arthritis. If so, it is likely that the antibody response in the spleen is stimulated predominantly by the parenterally administered antigen and not by autoantigens in the joint. We cannot deduce what immunological mechanism initiates joint damage but the localization of anti-CII antibody in articular cartilage (Stuart *et al.*, 1983) supports a pathogenic role for antibody. Furthermore, serum antibodies are of lower affinity in arthritic animals compared with those that fail to develop clinical symptoms of arthritis (Staines *et al.*, 1990). Low-affinity anti-collagen antibodies are cationic and are produced in lymph nodes draining the site of immunization (manuscript submitted).

In contrast to the popliteal nodes, the bone marrow was a significant contributor to anti-CII antibody production. There may be some local anatomical connection between the tibial marrow and the foot because significantly more antibody was made in the ipsilateral marrows of rats with unilateral disease. In this case, we conclude that antibody synthesis in the marrow is the consequence of regional arthritis: if, in contrast, it were to be the cause, then an explanation, so far elusive, would have to be found for the unequal systemic sensitization of the marrow in the two hind-limbs.

In comparison with the response in the brachial lymph nodes, the levels of antibody production in the spleen did not relate to clinical disease. The influence of the spleen was not neutral, however, because passive transfer of spleen cells from CII-immunized donors delayed disease onset and reduced its

severity in recipients subsequently challenged with CII. Conversely, splenectomy before intradermal immunization with a marginally arthritogenic dose of CII tended to cause an earlier onset of more severe disease. Splenectomy also led to an increase in serum antibody titre and increased antibody production in the bone marrow, shown here to relate to arthritis. Splenectomized mice immunized with CII also show a higher incidence of disease along with an increase in the serum antibody levels (Wooley *et al.*, 1989). In $(NZB \times NZW)F_1$ mice with murine lupus, splenectomy is known to lead to an increase in IgG class anti-DNA antibodies (Roubinian *et al.*, 1977) and in C3H mice injected with rat erythrocytes, the production of anti-erythrocyte autoantibodies is enhanced by splenectomy (Cox & Finlay-Jones, 1979). The role of the spleen is further demonstrated by the adoptive transfer of resistance to disease with purified splenic T cells from CII-immunized animals to normal recipients (Kresina & Moskowitz, 1985). There are, however, alternative or additional explanations for the role of the spleen in regulating CII arthritis and other autoimmune diseases. It could simply be a site of antigen catabolism; its removal therefore leaves more antigen available to stimulate autoreactive lymphocytes elsewhere. The spleen may also be an important site for the production of antibodies against CII, or immunoregulatory anti-idiotypic antibodies. Both types of antibody have been shown in passive transfer experiments to suppress disease (Staines *et al.*, 1981; Arita *et al.*, 1987).

All lymphoid tissues have been shown to contribute to the anti-collagen antibody response in rats immunized with CII injected intradermally, but the relative amounts of antibody that they make vary both between tissues and with time. Importantly they also vary according to the state of disease in individual animals, and a role for the spleen in suppressing antibody production has been identified. The bone marrow especially appears to reflect variations in local immunity according to disease in the sense that local production of antibody is highest near severely arthritic joints.

ACKNOWLEDGMENTS

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