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COLLAGEN-INDUCED POLYARTHRITIS IN RATS: A STUDY OF NATIVE TYPE II COLLAGEN FOR ADJUVANT ACTIVITY[1]

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Collagen-induced polyarthritis in rats is a new experimental model that shares clinical and histologic features with adjuvant arthritis. To determine whether collagen-induced arthritis is a form of adjuvant disease and to further exclude contamination of collagen with an adjuvant substance, native type II collagen was studied for adjuvant properties. IgM and IgG PFC activity and PBMC [^{3}H]TdR incorporation were studied in rats after injection with TNP-OA combined with IFA, IFA and CII, or CFA. In general, humoral and CMI responses to TNP-OA were lower in rats injected with IFA/CII compared with those with IFA; the presence of CII during primary immunization failed to significantly enhance PFC activity to TNP after a boost. CFA-injected rats gave maximal values in both studies. Mice pretreated with BII in the absence of oil gave PFC responses below control after sensitization with SRC. Furthermore, CII was unable to replace mycobacteria in the induction of EAE in rats and was devoid of mitogenic or polyclonal stimulatory properties. It is concluded that collagen-induced arthritis is a distinct entity from adjuvant arthritis and is dependent upon the unique immunogenicity of type II collagen in rats rather than upon an adjuvant effect.

Collagen-induced polyarthritis in rats is a new experimental model of inflammatory arthritis (1). It is unique in that it can be induced with type II collagen, a native protein, without the presence of bacterial adjuvants. In addition to being arthritogenic, type II collagen is a potent immunogen. Virtually all rats sensitized with type II collagen emulsified with incomplete Freund's adjuvant (IFA) develop a detectable humoral or cellular response, although both responses are significantly greater in 40 to 60% of rats that develop arthritis (2–4). Other collagen types including I, III, IV, and denatured α-chains, are nonarthritogenic, and produce weak humoral and cellular immune responses (1, 3–5).

Clinically, collagen-induced arthritis resembles adjuvant arthritis (6). Both disease models are restricted to the rat, require oil as a vehicle, appear after a latent interval after injection, involve peripheral joints, and share a similar histopathology (1, 6). In spite of these similarities, there are also dissimilar features. Adjuvant arthritis can be induced in virtually 100% of susceptible strains of rats and is usually severe (7); collagen-induced disease appears in only 40 to 60% of sensitized rats and is variable in severity. Spondylitis and extra-articular manifestations such as ear nodules and irtitis are common in adjuvant arthritis (8) but are infrequent or absent in collagen-injected rats (unpublished observation).

These observations suggested to us that native type II collagen should be investigated for adjuvant properties. The discovery of adjuvant activity in a native nonbacterial protein would be an exciting new observation. On the other hand, the absence of adjuvanticity would help to delineate these two models and further exclude the contamination of type II collagen with substances with adjuvant or possibly arthritogenic properties.

Three approaches were undertaken to determine whether native type II collagen has classical adjuvant activity (9), i.e., the ability to augment either humoral or cell-mediated immunity (CMI)[2] to an immunogen. First, an arthritogenic amount (100 μg/rat) of type II collagen was incorporated into emulsions of IFA in an attempt to "complete" incomplete Freund's adjuvant. Second, collagen was studied in the absence of an oil emulsion for adjuvant activity. Third, type II collagen was studied for mitogenic and polyclonal stimulatory activity, properties often associated with bacterial adjuvants.

MATERIALS AND METHODS

Animals. Outbred Wistar female rats, 100 to 125 g, were obtained from Charles Rivers Breeders (Wilmington, Mass.). Inbred Lewis rats, of the same sex and size, were obtained from Microbiological Associates (Bethesda, Md.) for use in studies of experimental allergic encephalomyelitis (EAE). Swiss female mice (15 to 20 g) were purchased from Harlan Laboratory (Indianapolis, Ind.); male C57BL/6 inbred mice were supplied by Charles Rivers courtesy of National Institutes of Health. Animals were fed standard laboratory chow.

Antigens. Chick type II collagen (CII) was prepared from

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[2] Abbreviations used in this paper: BII, bovine type II collagen; BP, myelin basic protein; CII, chick type II collagen; CMI, cell-mediated immunity; EAE, experimental allergic encephalomyelitis; SRC, sheep red cells; OA, ovalbumin; OA-HRC, ovalbumin-coated human red cells; PBMC, peripheral blood mononuclear cells; TNP-OA, trinitrophenylated ovalbumin; TNP-HRC, trinitrophenylated human red cells; TNP-RSA, trinitrophenylated rat serum albumin; TNP-SRC, trinitrophenylated sheep red cells; TNBS, trinitrobenzene sulfonic acid; HBSS, Hanks' balanced salt solution; [^{3}H]TdR, tritiated thymidine.

sterna of 3-week-old white leghorn chicks made lathyritic by the administration of β-aminoproprionitrile in the drinking water for 2 weeks. Tissue was pulverized with a freezer mill in liquid nitrogen (Spex Industries, Metuchen, N. J.) and extracted twice with 0.4 ionic strength potassium phosphate buffer, pH 7.6, for 24 hr. This and all subsequent steps were performed at 4°C. The residue was recovered by centrifugation and resuspended in 0.5 M acetic acid, pH was adjusted to 2.5 with formic acid, and residue was digested with pepsin (1 g per 20 g residue) for 48 hr. Both neutral salt and pepsin-extracted supernatants were dialyzed in 0.05 M Tris/0.2 M NaCl, pH 7.4, and passed through a DEAE-cellulose column that had been equilibrated with the same buffer. Collagen was precipitated from the effluent by adjusting the NaCl concentration to 3.5 M. Precipitates were dissolved in 0.5 M acetic acid and were reprecipitated with NaCl at a final concentration of 1.7 M. Collagen was reprecipitated from acetic acid solution three additional times. A final step was included to remove any contaminating type I collagen. Collagen solubilized in 0.2 M NaCl-Tris buffer was dialyzed in 0.05 M Tris/2.5 M NaCl, pH 7.4; precipitated type I collagen was removed by centrifugation, and the supernatant containing type II collagen was dialyzed in 0.1 M acetic acid and lyophylized.

Pepsin-extracted bovine type II collagen (BII) was prepared in a similar manner and has been previously described (5). Purity of collagen preparations was determined by amino acid analysis, carboxymethycellulose chromatography, and uronic acid analysis (10).

Trinitrophenylated ovalbumin (TNP-OA) and rat serum albumin (TNP-RSA) were prepared by reacting 15 mg of trinitrobenzene sulfonic acid (TNBS) with 100 mg of protein in 0.1 M carbonate-bicarbonate buffer, pH 9.5; unreacted TNBS was removed by filtering the reactants through a small column of AG-1-X8 resin (Bio-Rad, Richmond, Calif.). Hapten density determined by spectrophotometry indicated that each preparation contained seven TNP groups per carrier molecule. Guinea pig myelin basic protein (BP) was kindly provided by Dr. John Whitaker. Trinitrophenylated sheep red cells (TNP-SRC) were prepared by the method of Rittenberg and Pratt (11).

Sensitization. Wistar rats were immunized with TNP-OA in three groups. Group I was injected with TNP-OA antigen emulsified in IFA (Difco, Detroit, Mich.); Group II, with antigen, IFA, and CII; and Group III, with antigen, IFA, and heat-killed *Mycobacterium tuberculosis* (Ministry of Agriculture, Fisheries, and Food, Surrey, England). Each emulsion contained 1 volume of TNP-OA dissolved in water (2 mg/ml) and 10 volumes of IFA. Collagen was added to Group II by adding 9 volumes neutral salt-extracted CII (2.20 mg/ml) dissolved overnight in 0.1 M acetic acid at 4°C. Since the other two emulsions did not contain acid solubilized CII, equal volumes of 0.1 M acetic acid were added for control. Mycobacteria were incorporated to group III by grinding 5 mg of bacilli with 1 ml of IFA. TNP-OA plus solubilized CII or acetic acid were mixed and added to oil fraction; water-in-oil emulsions were prepared with a Virtis 23 homogenizer (Gardner, N. Y.) and maintained at 4°C. Each rat was injected intradermally with 0.05 ml (10 μg TNP-OA/rat) emulsion in the foot pad of each hind limb. Animals were exsanguinated by cardiac puncture at days 4, 7, 14, and 28. Blood and lymphoid tissues were studied for plaque-forming cell (PFC) activity, peripheral blood mononuclear cell (PMBC) for transformation, and plasma for hemagglutination. None of these activities could be detected at day 4; therefore, studies of this interval were not pursued. Significant PFC activity could not be detected in PBMC at any interval. The effects of collagen on an anamnestic response was investigated in a separate protocol. Three groups of Wistar rats were immunized as previously described and boosted i.p. at day 14 with 200 μg TNP-OA emulsified with IFA. Spleen PFC activity and plasma hemagglutination responses were studied 6 and 14 days later.

Emulsions containing BP were prepared in an identical manner, except pepsin-extracted CII dissolved in 0.1 M acetic acid and dialyzed in phosphate-buffered saline (PBS) was substituted for neutral salt extracted CII. Equal volumes of PBS were added to the other preparations. Lewis rats were injected with 0.1 ml of emulsions containing 50 μg BP intradermally in the right hind foot pad.

Swiss mice were injected i.p. with either 0.5 ml of PBS and PBS containing BII collagen (2 mg/ml). Twenty-four hours later both groups were sensitized i.p. with 0.2 ml of 10% SRC suspended in PBS. Four days later they were killed by cervical dislocation, and spleens were removed for SRC PFC activity.

Cell cultures. Spleen and draining lymph node were removed and teased apart with 18G needles in cold Hanks' balanced salt solution (HBSS). Particles were removed by aspirating the suspension through a gauze pledget. Cells were recovered by centrifugation at 800 rpm for 6 min. After two additional washes in HBSS, cells were resuspended in RPMI 1640 supplemented with 2 mM L-glutamine and counted, and viability was determined by trypan blue exclusion. Viability was 95% or greater. Cells assayed for PFC activity were kept at 4°C until used.

PBMC were obtained by Ficoll-Hypaque gradient centrifugation. PBMC tritiated thymidine ([^{3}H]TdR) incorporation studies were performed in microtiter plates and have been described previously in detail (5). Ovalbumin (OA) and TNP-RSA antigens were dissolved in RPMI and added to quadruplicate cultures at a final concentration of 22.5 μg/ml and 45 μg/ml, respectively.

Mouse (C57BL/6) spleen cell cultures for polyclonal stimulation studies were performed in duplicate in Falcon plastic Petri dishes (No. 3001, Oxnard, Calif.). Each dish contained 1×10^7 cells suspended in RPMI supplemented with 5% heat-inactivated fetal calf serum, 2 mM L-glutamine, 5×10^{-5} M 2-mercaptoethanol, penicillin (100 units/ml), streptomycin (100 μg/ml), and nucleic acids (25 μg/ml of cytosine, uridine, guanidine, and adenosine (Sigma, St. Louis, Mo.). CII or 0127:B8 *Escherichia coli* lipopolysaccharide (LPS; Sigma) in PBS were added at ten-fold dilutions to the cultures. Cell cultures were maintained in an environment of 5% CO_2, 95% air, high humidity at 37°C for 48 hr. Cells were washed twice in HBSS before PFC assay was performed.

PFC assay. PFC activity was determined in duplicate cultures by the method described by Jerne and Nordin (12). In rapid succession, a suspension containing 0.1 to 4×10^6 spleen or lymph node cells was added to tubes containing 2 ml of a 0.7% agarose solution (Sea Kem, Marine Colloid Products, Rockland, Maine) dissolved in MEM (No. 12-662 Microbiological Assoc.) and 100 μl of a 20% TNP-SRC suspension. The contents, maintained at 42°C, were mixed thoroughly and layered on plastic Petri dishes (Falcon No. 1007). IgG-producing PFC were developed by adding 100 μl of rabbit anti-rat IgG diluted 1:800 in HBSS. Plates were incubated 90 min in same environment as cell cultures, covered with 1.5 ml of SRC absorbed fresh frozen guinea pig sera diluted 1:10 in Veronal-buffered saline, and incubated for an additional 45 min. Complement was then poured off; the plates were left at room temperature for 2 to 3 hr, and developed plaques were counted

in indirect light. Since rabbit anti-rat IgG diluted at 1:800 gave optimal IgG plaque development and did not suppress IgM PFC activity, the number of IgG PFC was determined by subtracting direct PFC count from the total PFC count. Results are expressed as PFC/10^6 cells.

Hemagglutination. Hemagglutination was performed by using human A+ red cells (HRC) obtained from a single donor to which TNP had been conjugated by the method described for TNP-SRC. OA (40 mg/ml) was conjugated to human red cells (OA-HRC) with water soluble carbodiimide. This procedure and hemagglutination techniques have been previously reported (5).

Statistics. Student's *t*-test was used to compare the mean values of the study groups.

RESULTS

Effect of a second antigen on collagen-induced polyarthritis. The addition of a small amount of a second antigen, either 10 μg of TNP-OA or 50 μg of BP, to an arthritogenic dose of acid solubilized CII (100 μg/rat) had no appreciable effect upon the incidence or severity of collagen-induced arthritis. Eleven of 25 rats (44%) sensitized with collagen plus a second antigen developed arthritis when followed for a period of 20 days or longer. This figure is comparable to those of earlier reports (1, 2).

Primary humoral response to TNP-OA. The incorporation of type II collagen into an emulsion of TNP-OA and IFA failed to augment the primary immune responses as measured by the PFC response of draining lymph nodes or spleen and hemagglutinating activity. In Figure 1, IgM and IgG lymph node PFC responses are recorded for each group and time interval. It is apparent that maximal PFC values were found in animals injected with antigen and CFA. In this group the number of IgM PFC was significantly greater at day 7 ($p < 0.025$ *vs* IFA and $p < 0.05$ *vs* IFA/CII) and day 28 ($p < 0.001$ *vs* IFA and IFA/CII groups). IgG values were also significantly greater at day 7 ($p < 0.05$ *vs* IFA/CII group) and 28 ($p < 0.05$ *vs* IFA and $p < 0.025$ *vs* IFA/CII). On the contrary, the addition of collagen to the emulsion appeared to have a slight inhibitory effect. With only one exception, rats injected with antigen and IFA/CII produced PFC responses below those injected with antigen and IFA alone. These differences in mean values, however, were not statistically significant.

Little splenic IgM PFC activity was noted in any of the groups after primary sensitization. However, a significant IgG response was noted in CFA-injected animals at day 28, 140 ± 66 (± standard error) CFA *vs* 18 ± 5 IFA and 6 ± 2 IFA/CII ($p < 0.05$ and 0.025, respectively).

Circulating antibody, measured by hemagglutination of TNP-HRC, corresponded with PFC activity. These data are summarized in Table I. As noted earlier with the PFC response, combining CII with TNP-OA resulted in lower hemagglutination activity. This was notable at day 28, where mean titer from the IFA/CII group fell significantly below those of IFA and CFA ($p < 0.025$ and 0.001, respectively). Again, the greatest response was noted in rats sensitized to antigen with CFA. At day 7, rats injected with CFA yielded significantly higher titers

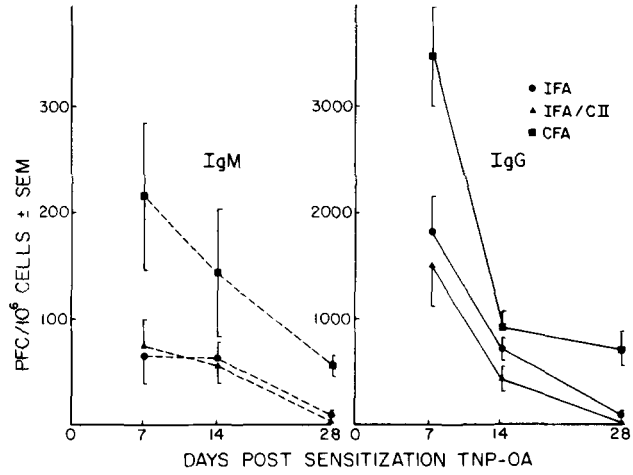

Figure 1. Time-course of rat lymph node IgM and IgG PFC response to TNP. Draining lymph nodes were removed at regular intervals after an intradermal injection of 10 μg TNP-OA was emulsified with IFA, IFA/CII (100 μg/rat), or CFA (250 μg mycobacteria per rat). Responses are mean values expressed as PFC/10^6 cells; bars represent the S.E.M. Each symbol represents a group of 12 to 16 wistar rats.

TABLE I

TNP and OA hemagglutinating activity of rats sensitized with TNP-OA emulsified with IFA, IFA/CII, or CFA[a]

Group	Primary Sensitization (Day) 7	14	20	28
		Anti-TNP antibody		
IFA	7.4 ± 1.5	11.2 ± 0.6	ND[b]	10.5 ± 2.6
IFA/CII	7.4 ± 2.6	11.1 ± 1.0	ND	8.3 ± 2.8 ($p < 0.025$)
CFA	9.0 ± 2.4 ($p < 0.05$)	11.8 ± 2.0	ND	11.5 ± 1.4
	IFA	Post boost[c]	12.1 ± 1.0	11.5 ± 0.5
	IFA/CII		11.8 ± 1.0	10.7 ± 1.1
	CFA		15.5 ± 3.3 ($p < 0.005$)	12.4 ± 0.5
		Anti-OA antibody		
IFA	0.9 ± 1.1	9.6 ± 1.6	ND[b]	10.4 ± 1.6
IFA/CII	1.1 ± 1.2	8.9 ± 1.5	ND	10.2 ± 1.0
CFA	1.3 ± 0.7	5.0 ± 3.2 ($p < 0.001$)	ND	6.5 ± 2.7 ($p < 0.001$)
	IFA	Post boost[c]	14.8 ± 2.7	13.2 ± 1.5
	IFA/CII		13.6 ± 1.4	13.3 ± 1.6
	CFA		10.8 ± 4.4	13.2 ± 2.7

[a] Groups of 10 to 16 wistar rats were sensitized with 10 μg TNP-OA emulsified with IFA, IFA and 200 μg CII, or CFA. Hemagglutinating activity was determined with TNP and OA-HRC and is expressed as the mean of $-\text{Log}_2$ titer ± S.D.

[b] Not done.

[c] In a separate study, groups of 6 to 8 rats were sensitized as described but given an i.p. boost (200 μg TNP-OA/IFA) at day 14 and studied 6 and 14 days later.

compared with the IFA and IFA/CII group ($p < 0.001$ and 0.025, respectively).

It is of interest that the addition of mycobacteria to IFA appears to increase hapten recognition. Titers to TNP were greatest in rats sensitized with CFA, whereas little hemagglutinating activity was detected to OA (Table I). On the contrary, rats sensitized with IFA or IFA/CII gave significantly greater anti-OA titers ($p < 0.001$ at day 14 and 28 *vs* CFA) and smaller TNP responses. Fourteen days after an i.p. boost (200 μg TNP-OA/IFA), titers to OA in rats sensitized with CFA reached levels comparable to those immunized without mycobacteria. The addition of CII did not affect hapten recognition.

The effect of collagen-induced arthritis on the immune response to TNP-OA was difficult to evaluate, partly due to the distribution of arthritic rats within the study groups and to the design of the experiment. Since rats were sacrificed at day 14, the mean day of onset being 14.8 ± 2.5 (standard deviation) days, the nonarthritic group would contain a number of animals that were destined to develop arthritis. On the contrary, if they were studied at a later date sufficient to segregate the two groups, the immune responses would be variably decreased, making comparison difficult. None the less, if the response of arthritic rats alone at day 14 are considered, no specific effect could be ascertained, since a range of high to low PFC responses and stimulation indices were observed.

Effects of collagen on antigen rechallenge. To evaluate the effects of CII on the humoral response after antigen rechallenge, three additional groups of rats were immunized according to the protocol described earlier. On day 14, each group was given an i.p. boost with 200 μg TNP-OA emulsified with IFA. Hemagglutination activity and splenic PFC were measured 6 and 14 days later. IgM and IgG PFC responses to TNP are shown in Figure 2. Brisk IgM and IgG PFC responses were noted 6 days after boost in rats primarily sensitized with CFA; however, no significant difference was found between the lower, predominantly IgG, responses of the IFA and IFA/CII groups. On day 28, 14 days after boosting, PFC activity had declined in all the groups, although some residual activity remained in the CFA group. Hemagglutination studies (Table I) gave similar results; CFA produced the greatest response to TNP ($p \leq 0.005$ *vs* IFA and IFA/CII at days 6 and 14 after boost.

Effects of collagen on CMI response. Ficoll-Hypaque-separated PBMC were studied *in vitro* for CMI to OA and TNP-RSA on days 7, 14, and 28 by [^{3}H]TdR incorporation. These data are shown in Figure 3. The mean stimulation indices to OA were lowest at each interval in rats sensitized with IFA/CII. These values were significantly below those of IFA on days 7 and 28 ($p < 0.05$ and 0.025, respectively). CFA gave the greatest stimulation at each time interval. Little or no stimulation was noted in TNP-RSA cultures.

Effect of collagen on the induction of EAE. EAE is an autoimmune disease that can be induced in rats by sensitization with purified BP (13). Although it is possible to induce EAE in rats with whole spinal cord extracts in the absence of paraffin oil or mycobacteria (14), both agents are required if purified BP is used to produce disease (15). This observation was utilized to see whether type II collagen could substitute for mycobacterial adjuvant. Typical EAE, manifested by hind limb paralysis, was observed in 10 out of 10 rats injected with purified BP and CFA. One of 10 rats injected with BP and IFA developed mild transient hind limb paresis; none of BP IFA/CII-sensitized rats developed clinical evidence of EAE. Histologic examination of brains from rats sensitized with CFA revealed typical vascular changes and demyelination. However, only mild perivascular histiocytic infiltrations were seen in one rat from the IFA and two from the IFA/CII group. It is apparent that CII cannot substitute for mycobacteria as an adjuvant in the induction of EAE.

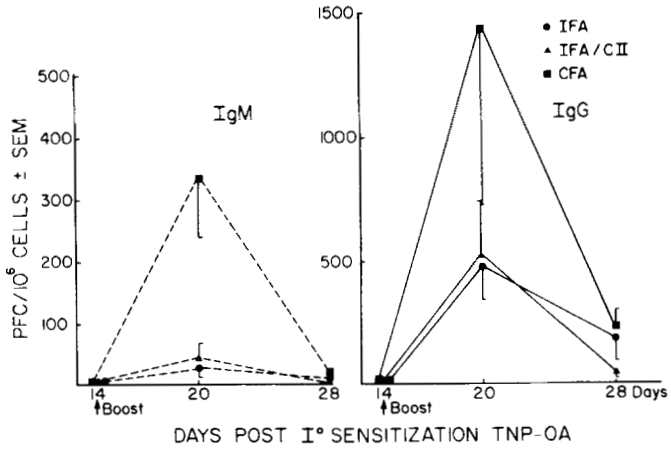

Figure 2. Rat spleen IgM and IgG PFC responses to TNP after an i.p. boost with 200 μg TNP-OA was emulsified with IFA. Rats were primarily immunized as described in Figure 1 but were boosted on day 14 and studied 6 and 14 days later; each symbol represents a group of eight rats. Results are mean values expressed as PFC/10^6 spleen cells ± S.E.M.

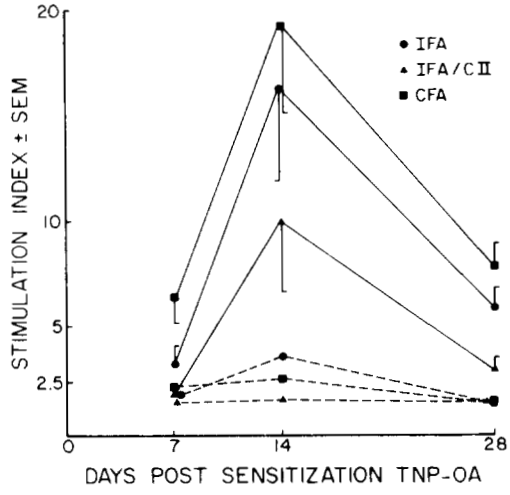

Figure 3. Time-course comparison of rat CMI to ovalbumin (*solid lines*) and TNP-RSA (*interrupted lines*) measured *in vitro* by PBMC [^{3}H]TdR incorporation. PBMC were obtained from animals used for PFC studies in Figure 1. Cells (1×10^6/ml) were cultured 96 hr in microtiter plates and pulsed with [^{3}H]TdR the final 6 hr. Results are expressed as means of stimulation indices (SI); bars represent S.E.M. SI = cpm, study culture/cpm control.

Effects of collagen on immune response in absence of oil. The above studies utilized neutral salt and pepsin-extracted CII in combination with IFA and did not eliminate the possibility that weak adjuvant activity might have been masked by the adjuvant property of IFA. To exclude this possibility, groups of six Swiss mice were injected i.p. with either 1 mg of pepsin-extracted BII solubilized in PBS or PBS alone as a control. Twenty-four hours later, both groups were sensitized with SRC, and the PFC response was studied 96 hr later. The mean response of mice pretreated with BII was 1257 ± 113 (± standard deviation) compared with 1910 ± 357 from control. The difference was not significant.

Evaluation of collagen as a polyclonal stimulator and mitogen. Spleen cells were pooled from normal C57BL/6 mice and

cultured with dilutions of LPS, CII, and heat-denatured CII. After 48 hr, cultured cells were recovered, washed, and studied for number, viability, and PFC activity to TNP-SRC and SRC. The PFC response is illustrated in Figure 4. A typical dose-response curve to TNP-SRC was obtained with LPS, a known polyclonal stimulator; however, no polyclonal activity could be demonstrated in either CII preparations. It is unlikely that CII was lymphotoxic, since the number of viable cells recovered were similar in LPS and CII cultures.

Mitogenic studies were performed by culturing spleen cells from normal Lewis rats with dilutions of CII. Parallel cultures were performed by using PBS as a negative control and concanavalin A and *E. coli* LPS as T and B cell mitogens. Results are summarized in Table II. After 72 hr of culture, significant [^{3}H]TdR incorporation was observed in cultures containing mitogens but not in those with CII or PBS.

DISCUSSION

Native type II collagen, when emulsified with IFA and injected into rats, demonstrates two distinct properties not found with other collagen types, i.e., strong immunogenicity and arthritogenicity. Although the humoral and cell-mediated responses are most evident in rats developing arthritis, they are also present in nonarthritic animals, although less intense (2–5). The incorporation of mycobacterial adjuvants (CFA) in the emulsions does little to augment the immune response to type II collagen when compared with emulsions prepared with IFA alone (2, 4). On the contrary, compared with type II collagen, types I and III even when combined with CFA give relatively weak immune responses and are incapable of inducing arthritis (1, 2, 4). The fact that type II collagen is arthritogenic in addition to the observation that both collagen and adjuvant-induced disease models share common clinical and histologic features raises the question whether type II collagen is a non-specific adjuvant and whether type II collagen-induced arthritis is a form of adjuvant arthritis. The similarity of these two models has also been suggested by reports that CMI to collagen can be demonstrated in rats with adjuvant arthritis (16, 17).

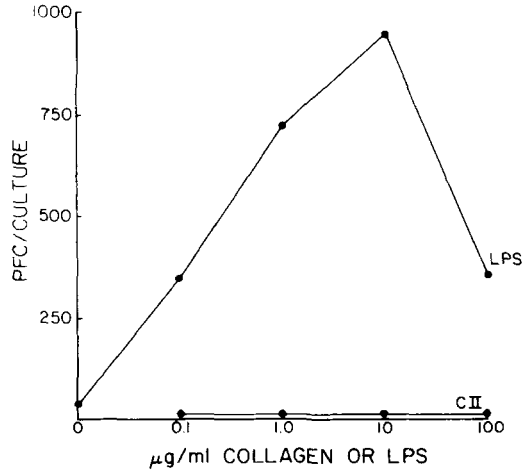

Figure 4. This figure represents one of three studies demonstrating the absence of polyclonal activity in native chick II collagen. Spleen cells were pooled from normal C57BL/6 mice and cultured 48 hr with CII or *E. coli* LPS. Polyclonal activity was determined by PFC activity to TNP-SRC.

Further support of the hypothesis that collagen might have adjuvant activity is indirectly suggested by two additional observations. Mozes *et al.* (18) have demonstrated in mice that type I collagen or a synthetic collagen-like copolymer were capable of making OA, a T-dependent antigen, behave in a T-independent manner. This property had been previously shown to exist in LPS, a known adjuvant (19). However, type II collagen was not studied, and type I collagen is not arthritogenic. Recently, Postlethwaite and Kang (20) have shown that native collagen types I, II, and III, their corresponding α-chains, and cyanogen bromide peptides are chemotactic for macrophages. Since these cells are essential in the generation of an immune response, their recruitment by collagen might, theoretically, facilitate an immune response.

However, the studies reported here fail to demonstrate classical adjuvant activity in three different preparations of type II collagen. When the immune response of rats injected with an arthritogenic amount of type II collagen, TNP-OA, and IFA was compared with those injected with TNP-OA and IFA alone, it was found that type II collagen not only lacked adjuvant properties but had a slight, consistent, inhibitory effect on the response to TNP-OA. This was apparent for both primary humoral and cell-mediated immunity. It does not appear that collagen simply delayed the immune response, since both responses were lower in the collagen group at each time interval studied. In contrast, rats sensitized with TNP-OA and CFA gave maximal humoral and cellular immune responses. Moreover, type II collagen was unable to augment the immune response to TNP on rechallenge or to substitute for mycobacteria in the induction of EAE. It also seems improbable that incorporating collagen with IFA might have masked a weak adjuvant response, since mice pretreated with PBS solubilized collagen before sensitization with SRC failed to produce a PFC response above that of controls.

The most plausible explanation for the slight observed inhib-

TABLE II

In vitro [^{3}H]TdR incorporation of rat spleen cells cultured with mitogen or collagen[a]

Rat	PBS	Con A	LPS	Chick Type II Collagen		
		10 μg/ml	10 μg/ml	1 μg/ml	10 μg/ml	50 μg/ml[b]
1	206 ± 30	108,403[c] ± 1,493 (526)[d]	1,543 ± 59 (7.5)	168 ± 80 (0.8)	353 ± 80 (1.7)	259 ± 24 (1.3)
2	268 ± 86	128,019 ± 2,875 (478)	3,769 ± 618 (14.1)	272 ± 46 (1.0)	172 ± 14 (0.6)	233 ± 40 (0.9)
3	312 ± 34	94,758 ± 3,138 (304)	3,024 ± 700 (9.7)	248 ± 24 (0.8)	287 ± 33 (0.4)	418 ± 65 (1.3)
4	183 ± 25	97,846 ± 2,193 (535)	1,294 ± 153 (7.1)	293 ± 36 (1.6)	261 ± 56 (1.4)	277 ± 28 (1.5)
Mean S.I. ± S.E.M.		461 ± 54	9.6 ± 1.6	1.1 ± 0.2	1.2 ± 0.2	1.3 ± 0.1

[a] Spleen cells (1×10^6 cells/ml) from normal Lewis rats were cultured in microtiter plates with PBS, concanavalin A, LPS, or native chick type II collagen for 72 hr. Cells were harvested after a 4-hr [^{3}H]TdR pulse.

[b] Final concentration of mitogen or collagen.

[c] Mean cpm ± S.E.M. of quadruplicate cultures.

[d] Stimulation index (S.I.) ± S.E.M. S.I. = cpm study culture/cpm control (PBS) culture.

itory response to TNP-OA injected with type II collagen is antigenic competition. This is supported by the observation that CII is a strong immunogen that also was found in another experiment to diminish similarly the response to TNP-OA emulsified in CFA. Other data have shown that CII does not activate nonspecific T cell suppression *in vitro*, thus excluding a nonspecific suppressive effect similar to that induced by concanavalin A.

It might be argued that type II collagen is neither an adjuvant nor arthritogenic in itself, but is contaminated by a substance with either or both properties. Proteoglycans might be considered as likely contaminants, since the collagen preparations were extracted from hyaline cartilage. However, these compounds are not arthritogenic (1) and could not be detected in collagen samples by uronic acid analysis. Furthermore, type II collagens prepared from hyaline cartilage and vitreous humor, sources differing in proteoglycan composition (21), have been shown to be equally immunogenic and arthritogenic (5).

Naturally occurring substances of bacterial origin that are known to be arthritogenic in the rat might possibly contaminate type II collagen preparations. Peptidoglycans and LPS are such ubiquitous bacterial products, small amounts of which are difficult to absolutely exclude as contaminants. However, both substances are mitogenic, polyclonal activators (22–24) and have classical adjuvant activity (25, 26), although only bacterial peptidoglycans are arthritogenic in the rat (27–29). LPS, on the contrary, have been shown in fact to inhibit the induction of adjuvant arthritis (30, 31). The absence of adjuvant and polyclonal activator properties of our CII preparations provide, therefore, some indirect evidence against significant amounts of bacterial contaminants.

Yet, the failute to demonstrate adjuvant activity in type II collagen does not absolutely exclude the potential presence of peptidoglycan contaminants, since Koga *et al.* (29) have shown recently that the adjuvant activity of mycobacterial-derived peptidoglycan can be separated from its arthritogenic activity. They found WAC, a water-soluble peptidoglycan derivative of Wax D, to be mildly arthritogenic in rats when administered with IFA but yet devoid of adjuvant activity. If WAC were combined with an acetylated subfraction of Wax D, which possessed only adjuvant activity, the severity of arthritis and its frequency increased markedly. However, it should be noted that intranodal injections with WAC at doses 100-fold greater than the minimum 2 μg intradermal arthritogenic dose of CII were required to induce even mild arthritis. Other points against the collagen-induced arthritis resulting from peptidoglycan contamination include the observation that many preparations of type II collagen from several species (1, 2) and different laboratories (4) have been consistently arthritogenic, whereas preparations of type I, III, and IV by using identical or similar reagents have not been (1, 5). Moreover, gentle heat denaturation at 42°C for 10 min, which results in uncoiling of the constituent α-chains, completely abolishes the capacity of type II collagen to induce either arthritis or a significant immune response. Peptidoglycan sequences or other contaminants would not likely be labile under these conditions.

In conclusion, it is proposed that collagen-induced arthritis is dependent upon an intrinsic property peculiar to helical type II collagen rather than a classical adjuvant effect or the presence of a contaminant. The nature of this property making type II collagen unique among collagens as an immunogen and arthritogen is not known. However, it is apparent that conformational integrity of the triple helix is an essential feature, since constituent α1(II) chains lack these properties (1).

There are several biochemical properties of type II collagen that distinguish it from types I and III. These include a greater content of hydroxylysine and hydroxylysine-linked carbohydrate groups (32). However, type IV collagen is nonarthritogenic (5) and shares these features. Therefore, it is unlikely that a quantitative difference alone in the molecular groups can explain the behavior of type II collagen. Another feature of type II collagen differing from types I and III is its relative resistance to cleavage by mammalian collagenase (33). This property might allow type II collagen to persist longer *in vivo* after injection, possibly increasing its immunogenicity or allowing it to disseminate to distant synovial sites where it might act later as an antigen. Although type IV collagen is also resistant to collagenase (34), it is susceptible to degradation by a nonspecific protease isolated from neutrophile granules, whereas type II collagen is not (35). Finally, type II collagen is unique in its anatomical distribution. Although other collagens are intimately associated with the circulation, type II collagen has been found exclusively in avascular compartments, including hyaline cartilage (32) and ocular vitreous humor (5). Although the relevance of these observations to understanding the unique properties of type II collagen or the pathogenesis of collagen-induced arthritis is not known, they should serve as subjects for future investigation.

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