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Adjuvant Oils Induce Arthritis in the DA Rat. I. Characterization of the Disease and Evidence for an Immunological Involvement

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An intradermal injection of Freund's incomplete adjuvant oil (FIA) without further additives was shown to induce erosive polyarthritis in dark Agouti (DA) rats, but not in Lewis rats. Histological examination revealed joint inflammation, first with polymorphonuclear cells and synovial hyperplasia, and subsequently, with multinucleated giant cells. Both constituents of FIA, mineral oil and Arlacel® A, as well as Pristane oil were arthritogenic, whereas vegetable oils were not. Re-administration of adjuvant oil after recovery failed to induce arthritis, thus making possible a role of specific immunity in this new form of arthritis in rats.

Introduction

Induction of chronic polyarthritis in certain rat strains can be provoked by a single injection of *Mycobacteria tuberculosis* (adjuvant arthritis; AA) [1] or by type II collagen (collagen-induced arthritis; CIA) [2] when mixed with an adjuvant oil. The investigations of these arthritis models have so far been focused mainly on the immune responses to the injected antigens, and the role of the oily adjuvant has been considered in more detail in only a few cases. In the most extensive study of this kind, Whitehouse *et al.* [3] examined many different oily vehicles for their co-arthritogenic and adjuvant activity together with *Mycobacteria tuberculosis* in rats, and found that

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several hydrocarbon compounds were very effective adjuvants. No indication was, however, provided in this paper of whether the oily compounds could induce arthritis without the addition of antigens.

In another line of research on plasmacytoma incidence in BALB/c mice, induced by intraperitoneal injection of pristane (2,6,10,14-tetramethylpentadecane), Potter and Wax [4] described an arthritis which occurred in the fore- and hind-paws of many of these animals. Further studies of this pristane-induced arthritis in mice have shown that the disease appears both to be mediated by immunological mechanisms [5] and shows genetic restriction [6]. In the case of pristane-induced arthritis, no particular attention has been paid to which oils are arthritogenic and which are not.

In this paper, we report that rats of the dark Agouti (DA) but not of the Lewis strain develop arthritis after injection of Freund's incomplete adjuvant (FIA) without any additive of antigen, and we have characterized this disease with regard to clinical and histopathological features, as well as certain immunological parameters.

Stimulated by these findings, we subsequently investigated other types of oils—mineral oils and vegetable oils—for their arthritogenicity in the DA rat.

Materials and methods

Animals

DA and Lewis rats of both sexes were obtained from inbred colonies at the animal unit at the Biomedical Centre in Uppsala. The DA rats were originally obtained from Bantin and Kingman Ltd, UK, and the Lewis rats from Moellegaard Laboratories, Denmark. In addition some DA rats were purchased from the Central Institute for Laboratory Animal Breeding, Hannover, Germany. The animals were used at 2 to 7 months-old and routine screening for pathogens gave negative results for Sendai virus, Corona virus, Pneumonia virus of mice and *Mycoplasma pulmonis*.

Oils

Arlacel® A (an emulsifier with mannide mono-oleat as its major component) and mineral oil (paraffin oil—a purified mixture of hydrocarbons obtained from petroleum) were purchased from Sigma Chemical Company, St. Louis, MO, USA. Pristane (2,6,10,14-tetramethylpentadecane) was purchased from Aldrich Chemical Company Inc., Milwaukee, WI, USA, and Freund's incomplete adjuvant (FIA) from Difco Laboratories, Detroit, MI, USA. Vegetable oils were purchased in a local drugstore.

Evaluation of oils for arthritogenicity

Each oil was assayed by injecting 200 µl intradermally into the base of the tail of one group of DA rats (except for Arlacel® A, where only 30 µl was injected, and mineral oil, where 170 µl was injected). FIA was in addition given to a group of Lewis rats. Animals were observed every second day for development of clinical signs of arthritis.

Oil-induced arthritis in DA rats

Clinical severity of arthritis was quantified according to the following grading scale: 1, detectable swelling in one joint; 2, swelling in two types of joints; 3, swelling in three types of joints,; 4, severe swelling of the entire paw. The maximum possible score for the four paws was therefore 16. Groups of rats injected with FIA were followed clinically, bled and killed at various times after injection of FIA. Inguinal lymph nodes were excised for *in vitro* assay and ankle joints were removed for histopathological examinations. Animals injected with other types of oils were followed only clinically.

Serological analysis

Sera were separated from blood samples and assayed for antibodies to type II collagen (CII) in an enzyme-linked immunosorbent assay (ELISA), as previously described [7]. Briefly, microtiter plates were coated overnight at 4°C with 50 μl/well of 10 μg/ml rat CII (prepared in our own laboratory) in phosphate-buffered saline (PBS). Dilutions of sera and washings were made in PBS containing 0.05% Tween-20. All tests were carried out in duplicate. Levels of anti-CII antibodies in a sample were quantified with goat-anti-rat IgG antibodies conjugated to alkaline phosphatase (Jackson Laboratories, West Grove, PA, USA). The subsequent quantification of bound enzyme was performed with a para-nitrophenol-containing substrate buffer in a Titertek multiscan spectrophotometer (Flow Laboratories, Eflab Oy, Helsinki, Finland).

T-cell proliferation assay

Lymph nodes from FIA-injected and normal DA rats were used to prepare individual single cell suspensions. These cells were seeded in 96-well microtiter plates (Nunc, Roskilde, Denmark) at a concentration of 2.5×10^5 cells/well in 200 μl DMEM, supplemented with glutamine, streptomycin, D-penicillin, HEPES, β-mercaptoethanol and 5% fetal calf serum. Cultures in triplicate were either incubated with 10 μg/ml rat CII, denatured (heated to 50°C for 30 min) rat CII, rat type I collagen (Sigma), mycobacterial 65 kDa heat shock protein (kindly provided by Dr J. van Embden, Bilthoven, The Netherlands) human proteoglycan (kindly provided by Prof. D. Heinegård, Lund, Sweden) or without any extra antigen. The cultures were placed in an incubator at 37°C for 4 days, and 1 μCi of [^{3}H]-thymidine (Amersham International plc, Amersham, UK) per well was added to the cultures during the last 16 h.

The incorporation of [^{3}H]-thymidine in the cells was measured in a liquid scintillation counter (Pharmacia, Wallac Oy, Turku, Finland) after harvesting of the cells with a Skatron cell harvester (Skatron AS, Lierbyen, Norway).

Histology

Ankle joints were fixed in 4% phosphate buffered formaldehyde and decalcified with 14% ethylenedinitrilotetraaceticacid (EDTA) (Titriplex®III, Merck, Darmstadt,

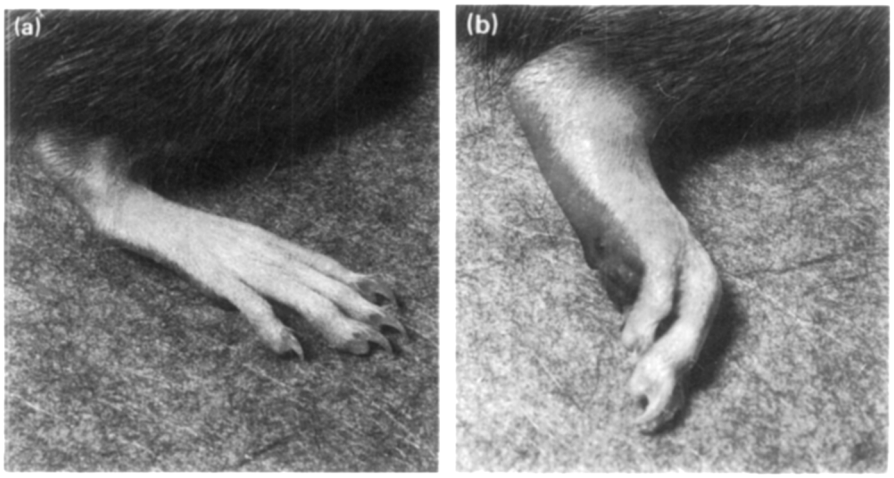

Figure 1. (a) Normal paw of a healthy DA rat. (b) Arthritic paw of a DA rat injected with FIA 24 days previously.

Germany) in 0.36 M NaOH for 2 weeks. After dehydration, clearing and impregnation in an autotechnicon tissue processor (2L Processor MK II, Shandon Southern Products Limited, Cheshire, England) the tissues were embedded in paraffin blocks. Sagittal sections were cut, mounted and stained with hematoxylin and eosin. Blood smears of healthy and FIA injected DA rats were air dried and stained with May Grünewald-Giemsa dyes (Merck), according to standard histological staining methdos. Two hundred white blood cells per animal were counted and the numbers of the different cell types were expressed as percentages.

Results

Characteristics of arthritis promoted by FIA in DA rats

Intradermal injection of a single dose of 200 µl FIA in DA rats induced polyarthritis in 100% of the animals (Figure 1, Table 1). Female and male rats responded in an almost identical way (Figure 2a and b). The initial signs of arthritis appeared 11–14 days after injection and were most often seen as symmetrical swelling of the metatarsophalangeal or ankle joints of the hind paws. The inflammation subsequently progressed to involve the entire hind paw and at this later stage the joints in the front paws often also became inflamed. No other organs showed macroscopic pathological changes. The arthritis declined and disappeared by about 45 days post-injection, without any detectable ankylosis or functional disorders. However, in some rats new bone formation was seen in the ankle joints. All rats showed a similar disease course with maximum arthritis scores varying between 4 and 12 for male and 6 and 14 for female rats.

To investigate whether re-injection with FIA would again give arthritis, DA rats that had recovered from oil-induced arthritis were challenged again with FIA, 39 days after the first injection. These rats did not develop any signs of arthritis (Table 1).

Table 1. *Incidence of arthritis in rats promoted by different types of oils*

Animals	Oil	Arthritis incidence
DA female	FIA	33/33
	Mineral	4/5
	Arlacel® A	5/5
	Pristane	5/5
DA female*	FIA	0/5
DA male	FIA	12/12
	Olive	0/5
	Sunflower	0/5
Lewis female	FIA	0/7
Lewis male	FIA	0/7

*Animals recovered from oil induced arthritis.

Analysis of susceptibility of oil-induced arthritis

FIA was also evaluated for its arthritogenicity in female and male Lewis rats. As seen in Table 1, none of the FIA injected Lewis rats developed any clinical signs of arthritis.

Oils tested for arthritogenicity

The two constituents of FIA, mineral oil and Arlacel® A, were separately evaluated for arthritogenicity. Mineral oil, constituting 85% of FIA, was injected at a dose of 170 µl into a group of DA rats. Another group of DA rats were injected with 30 µl of Arlacel® A, constituting 15% of FIA. The results shown in Table 1 demonstrate that both oils were able to induce arthritis in DA rats. Pristane oil (2,6,10,14-tetramethylpentadecane), often used in suspension with *Mycobacteria tuberculosis* when inducing AA in rats, was here tested alone at a dose of 200 µl for arthritogenicity. This type of oil also induced arthritis in DA rats (Table 1). The disease course for these oils was similar to that seen for FIA, concerning both its self-limiting nature and its severity.

Sunflower and olive oil were injected in a volume of 200 µl in separate groups of DA rats. Neither of these two vegetables oils were able to induce macroscopically detectable arthritis (Table 1).

Histopathological examinations of oil-induced arthritis

Ankle joints were obtained for histopathological evaluation at day 17 and 29 after injection of FIA. Joints taken at day 17 with clinically apparent arthritis showed minimal hyperplasia of the synovial lining layer (2–3 cell layers thick) and a massive infiltrate of polymorphonuclear cells in the synovial subintimal tissue and in the joint space (Figure 3a). On the other hand, mononuclear cells were rarely seen in the tissue

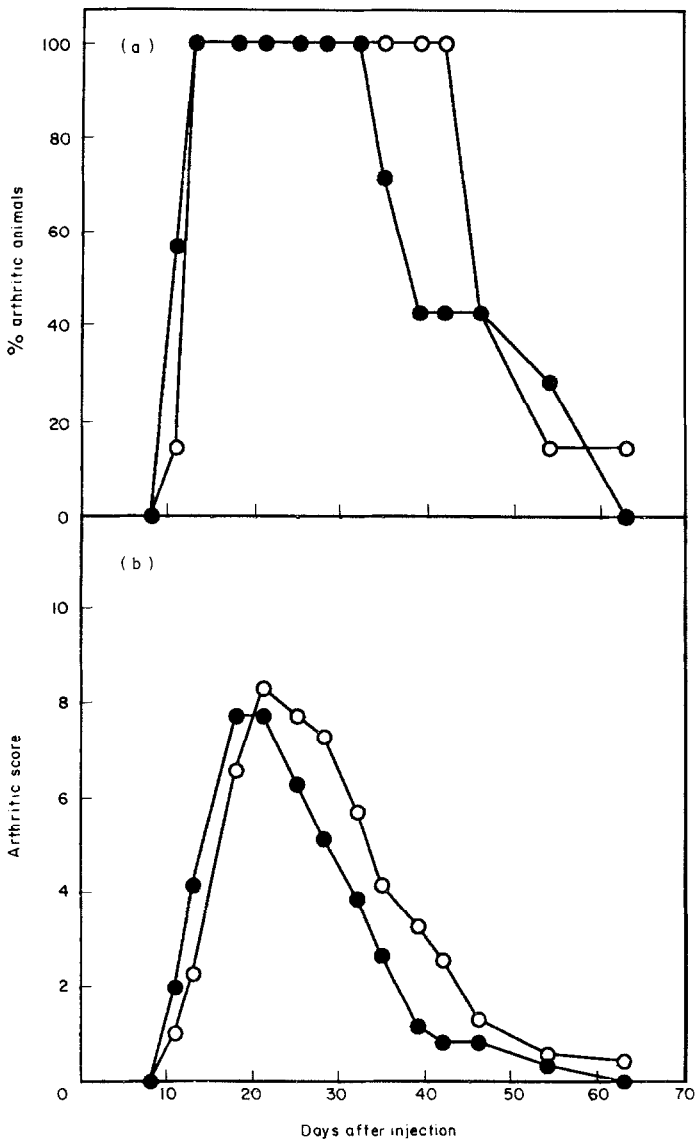

Figure 2. Oil-induced arthritis in DA rats. (○) female DA rats ($n=7$), (●) male DA rats ($n=7$). (a) Incidence of clinically apparent arthritis at different times after an intradermal injection of FIA. (b) Mean arthritic scores of arthritic rats at different times after an intradermal injection of FIA.

examined. In some joints an initial phase of marginal erosion in the subchondral bone was seen (Figure 3b). At day 29, both bone and cartilage destruction were apparent with pannus overlying cartilage (Figure 4a). The pannus tissue contained multinucleated giant cells as well as some mononculear and polymorphonuclear cells with the giant cells particularly dominant close to the sites of erosion (Figure 4b).

Blood samples collected on day 13 after injection of FIA showed an increase in the ratio of neutrophils to lymphocytes. The number of neutrophils increased from a

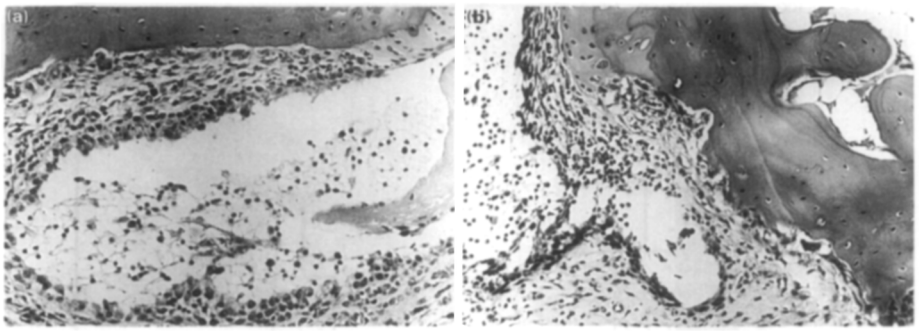

Figure 3. Hematoxylin and eosin stained sections from ankle joints of DA rats injected with FIA 17 days previously. (a) Minor hyperplasia of the synovial lining layer with infiltrate of polymorphonuclear cells in the synovial subintimal tissue and within the joint space (original magnification × 200, 35% reproduction). (b) Marginal erosion of the subchondral bone, associated with polymorphonuclear cell infiltration of the synovia and joint space (original magnification × 200, 35% reproduction).

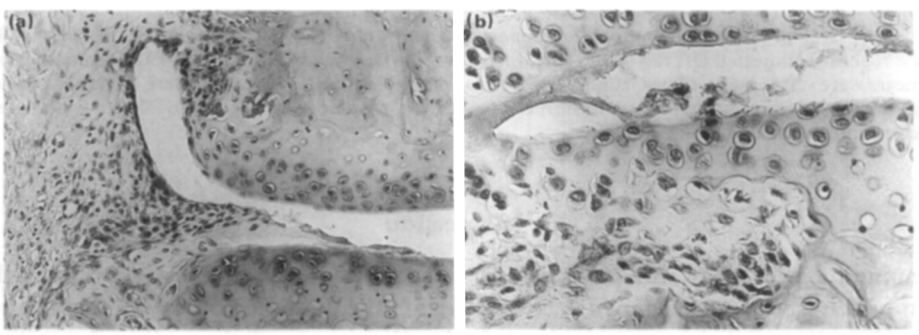

Figure 4. Hematoxylin and eosin stained sections from ankle joints of DA rats injected with FIA 29 days previously. (a) Marginal erosions of the subchondral bone and cartilage with formation of pannus overlying cartilage (original magnification × 200, 35% reproduction). (b) Erosion site of cartilage and underlying bone characterized by multinucleated giant cells (original magnification × 400, 35% reproduction).

mean of 8% in healthy DA rats to a mean of 42% in FIA injected DA rats. The number of monocytes varied from 7% in healthy DA rats to 8% in the FIA injected group (Figure 5).

Cellular and humoral responses in oil-induced arthritis

Lymph nodes from individual DA rats obtained at days 11, 17 and 24 after FIA injection (five rats at each time) were used to prepare single cell suspension, and the lymph node cells were then tested for specificity *in vitro*. Native or denatured rat CII, rat CI, 65 kDa heat shock protein and human proteoglycan were used as antigens in a T-cell proliferation assay. The cellular reactivity at day 11, 17 or 24 after injection did not differ from the responses to the same antigens seen with lymph node cells from six non-injected DA rats.

Individual serum samples from these rats at days 11, 17 and 24 after FIA injection were examined for anti-rat CII antibodies in an ELISA assay. None of the sera tested showed any reactivity to rat CII.

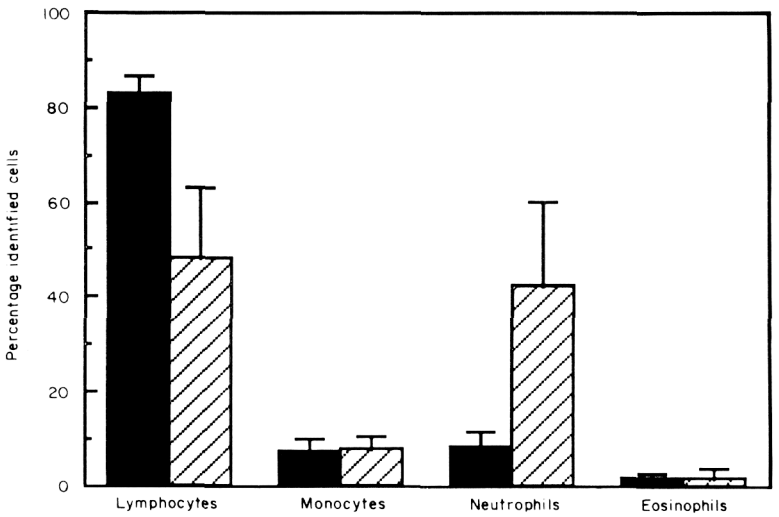

Figure 5. The ratios of lymphocytes : monocytes : neutrophils : eosinophils in blood of healthy (■) ($n=7$) and FIA injected DA rats (▨) ($n=7$). Blood samples were taken 13 days after FIA injection. Blood smears were stained with May-Grünewald-Giemsa and 200 leucocytes per smear were identified by their morphological features and counted. The mean percentage of each cell type is shown. Note the increase of neutrophils in the FIA injected animals.

Discussion

Immunological adjuvants have gained widespread acceptance for enhancing the immune response to certain injected antigens in the induction of experimental autoimmune diseases in laboratory animals (polyarthritis, encephalomyelitis, thyroiditis, etc.) [1–2], [8–9]. In certain rat strains experimental arthritis can only be induced by a combination of *Mycobacteria tuberculosum* and a suitable oily vehicle, such as AA; administering the mycobacteria in a saline suspension, for example, fails to induce arthritis [10]. The same applies to collagen-induced arthritis which is most often induced by an emulsion of type II collagen and a suitable adjuvant [2].

In this study we describe a polyarthritis found in the DA rat strain after a single injection of FIA, a commonly used adjuvant. This finding has not been previously reported although a similar pristane oil-induced arthritis in mice has been described [4–6]. However, no evaluation of the arthritogenicity of oils other than pristane has been performed in mice.

In carrying out a characterization in our DA rats, both constituents of FIA, mineral oil and Arlacel® A were shown to induce arthritis independently of each other. Furthermore, pristane oil could also induce arthritis, whereas neither of two tested vegetable oils were arthritogenic. These results indicate that the arthritogenic compounds may possess properties unique to particular classes of chemical compounds. These findings also indicate that the capacity of an oil alone to induce arthritis in DA rats parallels the capacity of this oil to act as a good adjuvant together with other agents, for example in inducing AA [3]. Thus, Whitehouse [11] reported that linear alkanes, many esters and triglycerides of fatty acids with a chain length of C12 or more and acetates of vitamins E, K and A are compounds with common adjuvant

activity. In both cases the exact function of the oily vehicle, the adjuvant is unknown, although FIA is known to stimulate macrophages, promote uptake of antigen and enhance antibody formation, but not to induce cell-mediated immunity [12].

With respect to the mechanisms involved in the development of oil induced arthritis in DA rats, histological examination of the inflamed joints revealed early infiltration of polymorphonuclear cells, whih was later followed by the appearance of multinucleated giant cells and some mononuclear cells. These observations are compatible with the presence of an early acute type of inflammation, which is followed by a more chronic phase where the presence of multinucleated giant cells indicates pronounced macrophage activation. Involvement of lymphocytes, i.e., specific immunity, in the development of oil-induced arthritis is also indicated by the facts that the arthritis is first seen about 14 days after injection of oil, and that arthritis cannot be induced after a second injection of oil; a similar specific resistance to re-induction of disease is observed in many autoimmune diseases which depend on reactivity to defined autoantigens [see for example 13]. The fact that repeated injections of an antigen in FIA, for example booster immunizations, do not cause general immunosuppression supports the explanation that the observed resistance to disease is dependant on specific changes in the immune system, rather than unspecific suppression.

The observation that only DA but not Lewis rats are susceptible to arthritis shows that genetic factors are of importance for the disease, and also why this type of oil-induced arthritis has not been previously reported; DA rats have rarely been used in studies of inflammatory arthritis.

The main question is, however, how to explain why a single injection of oil at the base of the tail can induce a destructive and probably immunologically mediated inflammation in the joints, but not elsewhere. One possibility, previously discussed by Rook *et al.* [14], would be that increased levels of IL-6 triggered by the oil injection induce formation of agalactosyl IgG, which in turn has been associated with development of arthritis. This and other possibilities should now be open to study in the present model.

An obvious problem is the definition of the relationship between this oil-induced arthritis and the previously extensively studied conditions where arthritis is induced after immunization by mycobacteria or type II collagen mixed in oil. With respect to the latter, both AA and CIA in DA rats differ from the oil-induced arthritis in being more destructive (both AA and CIA) and in being more chronic and involving more peripheral joints (CIA) [15]. In these cases it thus appears that induction of specific immune reactions to mycobacteria or type II collagen in DA rats adds particular clinical features to the arthritis already induced by the oil. Notably, collagen autoimmunity which was induced after immunization with type II collagen in olive oil did not cause arthritis in DA rats, thus further emphasizing the role of adjuvant in inflammatory diseases induced after injection of specific autoantigens (Kleinau *et al.*, to be published).

The extent to which specific immune reactions, for example to previously known arthritogenic autoantigens, contribute to the arthritis induced by oil only, remains unknown. By the conventional methods used in the paper no reactivities could, for example, be demonstrated, either against type II collagen or to hsp 65 kDa. For both these and other specific 'candidate antigens' we believe, however, that specific

immunotherapy which can selectively downregulate a potentially arthritogenic reactivity must be tested in order to prove or disprove its pathogenetic importance.

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References

1. Pearson, C. M. 1956. Development of arthritis, periarthritis, and periostitis in rats given adjuvant. *Proc. Soc. Exp. Biol. Med.* **91:** 95–101
2. Trentham, D. E., A. S. Townes, and A. H. Kang. 1977. Autoimmunity to type II collagen: An experimental model of arthritis. *J. Exp. Med.* **146:** 857–868
3. Whitehouse, M. W., K. J. Orr, F. W. J. Beck, and C. M. Pearson. 1974. Freund's adjuvants: Relationship of arthritogenicity and adjuvanticity in rats to vehicle composition. *Immunology* **27:** 311–330
4. Potter, M. and J. S. Wax. 1981. Genetics of susceptibility to pristane-induced plasmacytomas in BALB/cAn: Reduced susceptibility in BALB/cJ with a brief description of pristane-induced arthritis. *J. Immunol.* **127:** 1591–1959
5. Bedwell, A. E., C. J. Elson, and C. E. Hinton. 1987. Immunological involvement in the pathogenesis of pristane-induced arthritis. *Scand J. Immunol.* **25:** 393–398
6. Wooley, P. H., J. R. Seilbold, J. D. Whalen, and J. M. Chapdelaine. 1989. Pristane-induced arthritis. The immunological and genetic features of an experimental murine model of autoimmune disease. *Arthritis Rheum.* **32:** 1022–1030
7. Kleinau, S., P. Larsson, J. Björk, R. Holmdahl, and L. Klareskog. 1989. Linomide, a new immunomodulatory drug shows different effects on homologous versus heterologous collagen-induced arthritis in rats. *Clin. Exp. Immunol.* **78:** 138–142
8. Kies, M. W. and E. C. Alvord. 1959. *Allergic encephalomyelitis.* Charles C Thomas Publishers, Springfield, IL.
9. Vladutin, A. O. and N. R. Rose. 1971. Autoimmune murine thyroiditis relation to histocompatibility (H-2) type. *Science* **174:** 1137–1139
10. Ward, J. R. and R. S. Jones. 1962. Studies on adjuvant-induced polyarthritis in rats. I. Adjuvant composition, route of injection, and removal of depot site. *Arthritis Rheum.* **5:** 557–564
11. Whitehouse, M. W. 1977. The chemical nature of adjuvants. In *Immunochemistry: An advanced textbook.* L. E. Glynn and M. W. Steward, eds. Wiley. pp. 571–605
12. Waksman, B. H. 1980. Adjuvants and immune regulation by lymphoid cells. In *Immunostimulation.* Springer Seminars in Immunopathology 2. L. Chedid, P. A. Miescher and H. J. Mueller-Eberhard, eds. Springer-Verlag, Berlin, Heidelberg and New York. pp. 4–33
13. Lider, O., T. Reshef, E. Beraus, A. Ben-Nun, and I. R. Cohen. 1988. Anti-idiotypic network induced by T cell vaccination against experimental autoimmune encephalomyelitis. *Science* **239:** 181–183
14. Rook, G., S. Thompson, M. Buckley, C. Elson, R. Brealey, C. Lambert, T. White, and T. Rademacher. 1991. The role of oil and agalactosyl IgG in the induction of arthritis in rodent models. *Eur J. Immunol.* **21:** 1027–1032
15. Larsson, P., S. Kleinau, R. Holmdahl, and L. Klareskog. 1990. Homologous type II collagen-induced arthritis in rats; Characterization of the disease and demonstration of clinically distinct forms of arthritis in two strains of rats after immunization with the same collagen preparation. *Arthritis Rheum.* **33:** 693–701