Immunology 1983 **49** 511

The successful use of asialylated IgG as an immunogen and arthritogen in the rabbit

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Accepted for publication 11 February 1983

Summary. Joint lesions, closely resembling the main features of those seen in rheumatoid patients, were produced by intra-articular injections of asialylated homologous IgG into presensitized rabbits. The inflammatory changes were characterized by areas of extremely dense chronic inflammatory cell infiltration, where the lymphocytes were often aggregated into lymphoid follicles. There were also signs of involvement of the contralateral, saline-injected knee. Formation of an experimental rheumatoid factor-like antibody, detected by its ability to agglutinate sheep erythrocytes sensitized with baboon IgG, was also demonstrated. In addition, the rabbits developed other manifestations associated with rheumatoid arthritis, namely increases in erythrocyte sedimentation rate, serum haptoglobin concentration and joint size.

INTRODUCTION

Investigations into the aetiology and pathogenesis of human arthritis have been aided by the development of animal models in which the development of joint lesions can be induced experimentally. A variety of immunological and non-immunological methods has been used to induce arthritis in normal laboratory animals, although the model originally developed by Dumonde & Glynn (1962) has proved to be one of the most successful. These authors found that, following immunization of rabbits with fibrin in Freund's complete adjuvant, a single intra-articular injection of the same antigen into the knee-joint produced lesions which closely resembled, in their main features, those of rheumatoid arthritis in man. However, this model fails to express the systemic manifestations normally seen in rheumatoid arthritis, namely rheumatoid factors and immune complexes, which may also explain why there is a lack of progression of the experimentally induced disease to other uninjected joints.

The aim of the present work was to induce an arthritis in rabbits which exhibited joint lesions as severe as those observed in other rabbit models and also produced a circulating experimental rheumatoid factor. The experimental procedure used was similar to that of Dumonde & Glynn, except that asialylated IgG was the administered antigen as there have already been indications that asialylation of IgG can render it immunogenic in homologous hosts (Duc Dodon & Quash, 1981). Evidence that alteration of gammaglobulin can lead to the exposure of previously hidden determinants in the Fc portion of the IgG molecule was originally presented by Henney & Stanworth (1965), although the way in which homologous IgG becomes immunogenic has still to be elucidated. Moreover, it is interesting to note that the site of attachment of carbohydrate residues is in the

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0019-2805/83/0700-0511$02.00

same area of the Fc fragment (in the neighbourhood of the hinge region) as some of the reactive determinant sites (Stanworth, 1966; Hunneyball & Stanworth, 1976). Thus, by removing the terminal sialic acid residues, asialylation could possibly influence the secondary structure of IgG and so alter its immunogenicity.

MATERIALS AND METHODS

Materials

Neuraminidase (type V from *Clostridium perfringens*) was obtained from Sigma Chemicals Co., Poole, Dorset. Enzacryl-AH was purchased from Koch-Light Laboratories, Haverhill, Suffolk and diethylaminoethyl (DEAE) cellulose from Whatman, Kent. New Zealand white rabbits of either sex, weighing 2·5–3·5 kg and aged 3–5 months were used in all the experiments described.

Preparation of native and asialylated rabbit IgG

Rabbit IgG was isolated from pooled rabbit serum by a combination of precipitation with 33% saturated ammonium sulphate followed by batch ion-exchange chromatography on DEAE cellulose (Whatman DE52) according to Stanworth (1960). Any aggregated material was removed from the native rabbit IgG solution by centrifugation at 60,000 r.p.m. (400,000 ***g***) for 2·5 hr at 4° in an M.S.E. Superspeed 65 ultracentrifuge using an M.S.E. 6 × 4·2 ml (Titanium) swing-out rotor. After centrifugation, the top 2/3 of the supernatant were removed and kept as 'native IgG', the remainder being discarded.

Asialylation of the rabbit IgG was achieved by the method described by Duc Dodon & Quash (1981). This involved immobilizing the enzyme neuraminidase by covalent binding to a solid support, e.g. Enzacryl-AH, to prevent contamination of the final IgG preparation. Activation of the Enzacryl-AH with dilute nitrous acid generates acid azide groups (from the acyl hydrazide group $NHNH_2$) which are effective in coupling with neuraminidase at the side chain amino groups of lysine and the free amino terminals of the protein chains. A solution containing 20 mg of the purified rabbit IgG was mixed at 37° for 16 hr with 500 mg of the Enzacryl-Nase. The Enzacryl-Nase was then eliminated by filtration with 0·1 M borate buffer, pH 8·1, containing 1·0 M NaCl. The eluate contained the asialylated IgG which, after ultra-filtration, was dialysed extensively against 0·01 M phosphate buffer, pH 8, at 4°.

Sialic acid remaining on the IgG was determined by the fluorimetric assay developed by Murayama *et al.* (1976), in which free sialic acid reacts with pyridine to give fluorescent compounds. Fluorescence intensity of the solution was determined with an Aminco-Bowman spectrofluorometer, using light of 395 nm wavelength for excitation and 470 nm for emission.

Immunization of rabbits

Immunization was achieved by subcutaneous injection, into three or four sites of the interscapular region, of 10 mg of either asialylated or untreated IgG in 0·5 ml sterile saline emulsified with an equal volume of Freund's complete adjuvant (FCA; Difco). The injections were repeated twice more at 14-day intervals.

Induction of arthritis

When the animals had developed delayed hypersensitivity to the antigen, the right knee was aseptically injected with 10 mg of the same IgG preparation as used to immunize the rabbit, in 0·5 ml physiological saline. As controls, the left knees were injected with the same volume of sterile saline.

Measurements of the immune response

Haemagglutination analysis. The anti-human IgG component of human rheumatoid factor can be quantified by haemagglutination of sheep erythrocytes sensitized with baboon anti-sheep erythrocyte IgG antibody (Stewart, Hunneyball & Stanworth, 1975). The circulating antibody produced experimentally in the rabbits was measured in a similar manner. Haemagglutination analyses were performed in the conical-bottomed wells of a Multi-Well Disposo Tray (Linbro Flow Laboratories). Sera were decomplemented by heating at 56° for 20 min prior to use.

Erythrocyte-sedimentation rate (ESR). Two millilitres of blood were taken into trisodium citrate solution (3·1%) for Westergren ESR measurements. The erythrocyte sedimentation rate was measured over 1 hr at room temperature, at various times throughout the experiment.

Knee measurements. The width of both knee-joints of each rabbit was measured with engineer's callipers. The degree of swelling was expressed as the ratio of the width of the antigen-injected knee-joint to that of the control saline-injected knee-joint. Measurements were taken at regular intervals.

Histological assessment of arthritis. The intensity of inflammation of the synovium was graded in a similar manner to that described by Consden *et al.* (1971) on a 0 to 4 scale, 0 being normal synovium, whilst 4 is complete involvement of the entire synovium with very dense cellular infiltration.

RESULTS

Delayed-hypersensitivity measurements

The ability of all rabbits to give a delayed-hypersensitivity response to either untreated or asialylated IgG was demonstrated 6 weeks after the first immunization injection (Fig. 1). This involved intradermal injections with 10, 30 and 50 μg of both IgG preparations in 0·1 ml sterile saline.

Haemagglutination analysis

The circulating antibody produced in the rabbits immunized with asialylated IgG in FCA was capable of agglutinating sheep erythrocytes sensitized with baboon IgG (Fig. 2). A convincing anti-gammaglobulin titre was observed 5 weeks after the initial immunization, but even at day 10 after the first injection of asialylated rabbit IgG, a low titre was recorded. The rabbits immunized with the untreated IgG failed to produce a haemagglutinating antibody. This implies that these animals were tolerant to the untreated IgG, which had no determinants capable of eliciting an immune response in members of the same species.

Neither group of rabbits produced a circulating antibody capable of agglutinating sheep erythrocytes sensitized with normal rabbit IgG.

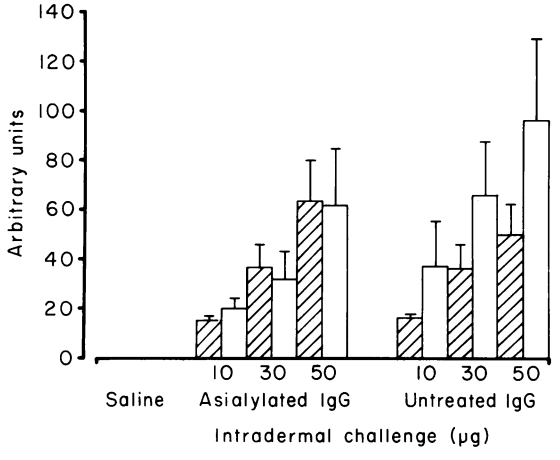

Figure 1. Twenty-four hour delayed cutaneous hypersensitivity reactions to intradermal injections of 10, 30 and 50 μg doses of asialylated and untreated rabbit IgG in rabbits immunized on days 1, 16 and 30 with 10 mg of either untreated IgG (■) or asialylated IgG (□) in FCA subcutaneously. Each bar represents the standard error of the mean (SEM) of 4–5 animals.

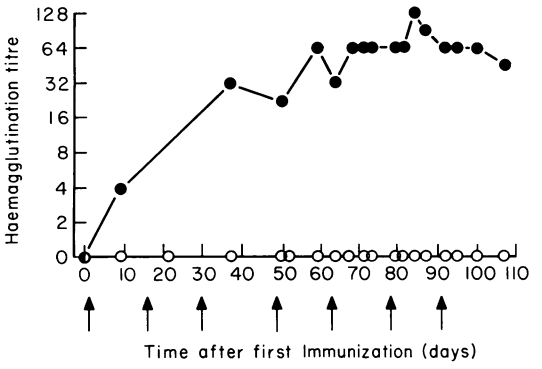

Figure 2. Circulating antibody response, as measured by haemagglutination of sheep erythrocytes sensitized with baboon IgG, in rabbits immunized on days 1, 16 and 30 with 10 mg of either untreated IgG (○) or asialylated IgG (●) in FCA subcutaneously. Arthritis was induced by four intra-articular injections into the right knee-joint on days 49, 63, 78 and 91 with the same IgG preparations as used for immunization. The left knee-joints received the same volume of sterile saline. Each point represents the mean of 4–5 animals.

Erythrocyte-sedimentation rate

An increase in the ESR was observed in both groups of rabbits as a result of the immunization (Fig. 3). Although the mean values shown by the rabbits that had been treated with asialylated IgG increased noticeably after the intra-articular injections, the standard errors also increased, so that the difference was insignificant statistically.

Knee-width measurements

The ratio of the width of the antigen-injected knee (right) to that of the saline-injected knee (left), R/L, is shown in Fig. 4. A statistically significant increase in the right-knee measurement was observed in both groups of rabbits as a result of each intra-articular injection, although the rabbits that received the injections of asialylated IgG showed the more severe joint swelling.

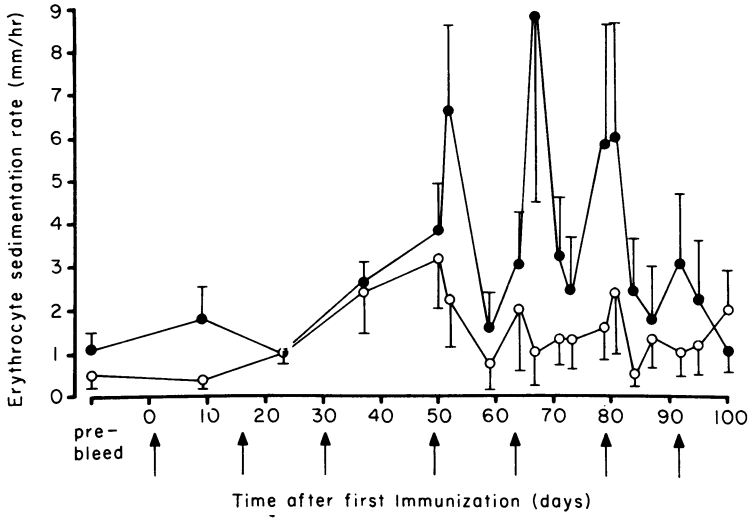

Figure 3. Changes in erythrocyte sedimentation rate in rabbits immunized on days 1, 16 and 30 with 10 mg of either untreated IgG (○) or asialylated IgG (●) in FCA subcutaneously. Arthritis was induced by four intra-articular injections into the right knee-joint on days 49, 63, 78 and 91 with the same IgG preparation as used for immunization. The left knee-joints received the same volume of sterile saline. Results are presented as the mean ± SEM of 4–5 animals.

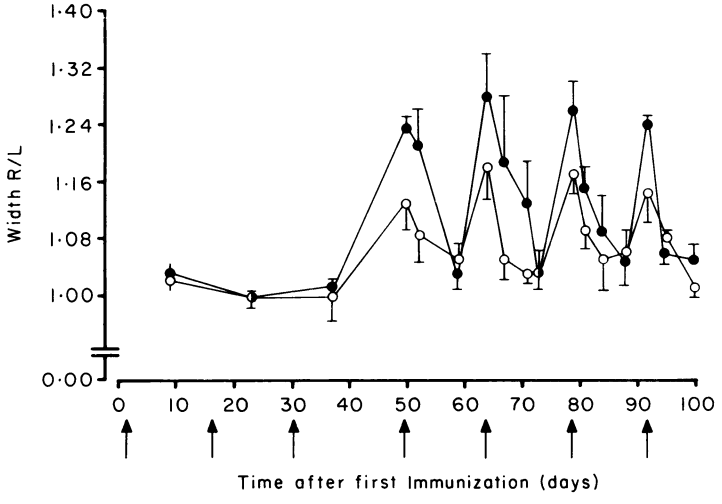

Figure 4. The ratio of the right-knee to left-knee widths (R/L). Arthritis was induced by four intra-articular injections into the right knee joint on days 49, 63, 78 and 91 with 10 mg of the same IgG preparation as used for immunization; untreated IgG (○) or asialylated IgG (●). As controls the left knee-joints received the same volume of sterile saline (0·5 ml). Results are presented as the mean ± SEM of 4–5 animals.

Terminal histopathological assessment

The rabbits were all killed 8–9 weeks after the first intra-articular injection (2 weeks after the fourth and final intra-articular injection).

At the macroscopic level, severe changes were observed in many of the antigen-injected knees. These changes usually consisted of enlarged fat pads, which frequently extended into the intercondylar fossa, covering the cruciate ligaments, and thus making removal of the intact fat pad difficult. Both the infrapatellar and the suprapatellar fat pads of the right, antigen-injected, knee-joints were often discoloured and hyperaemic in comparison with those of the left, saline-injected, knee-joint.

Microscopic examination of the infrapatellar fat pads revealed extensive inflammatory changes in the antigen-injected knee-joints of the rabbits with asialylated IgG. There was evidence of fibroblasts, fibrocytes and collagen depositoin throughout the whole of the synovium, the extent of this fibrosis being graded with the more severe deposition occurring closest to the synovial lining cells (Fig. 5). Hyperplasia of the synovial lining cells was severe with a clear boundary between this and the extensive chronic inflammatory cellular infiltration, typified by lymphocytes with a more diffuse infiltration of plasma cells. The infiltrating cells were often aggregated into follicles consisting mainly of lymphocytic cells. There was also much perivascular infiltration and fibromatosis (thickening of the blood vessel walls). Several of the saline-injected left knee-joints of these same animals showed mild inflammatory changes (Fig. 6) in that the fat pads were slightly fibrosed with signs of cellular infiltration, hyperplasia of the synovial lining cells and the beginnings of villus formation.

The rabbits which received four intra-articular injections of the untreated IgG exhibited features which were not as severe as those shown by the rabbits treated with asialylated IgG, the changes being indicative of a milder inflammatory response. Cellular infiltration occurred throughout the synovium and there were also areas of aggregated lymphocytes and other mononuclear cells.

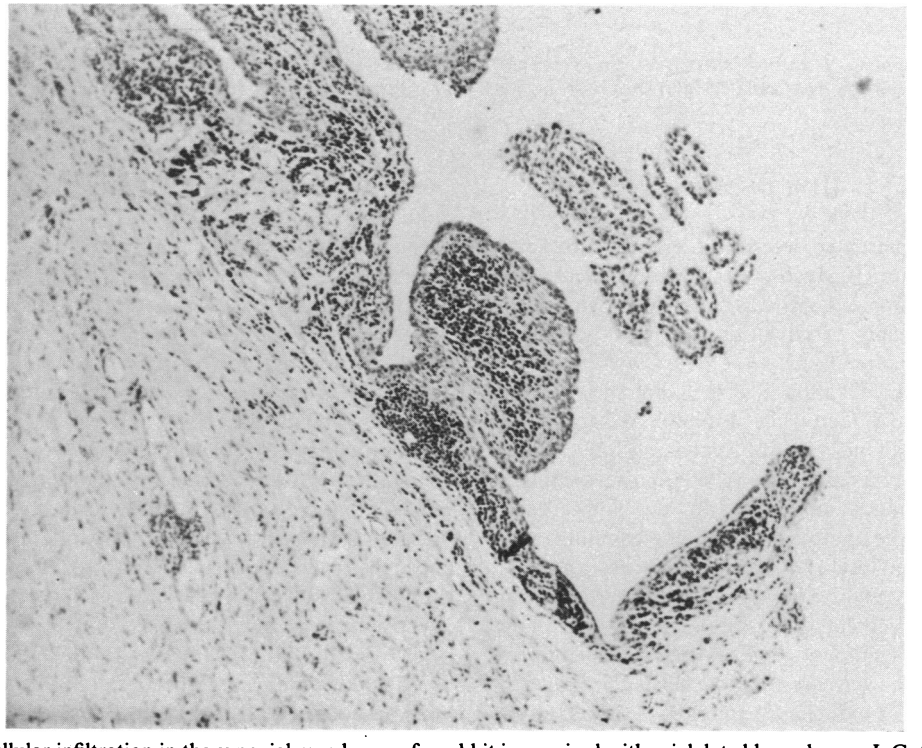

Figure 5. Cellular infiltration in the synovial membrane of a rabbit immunized with asialylated homologous IgG, followed by four intra-articular injections with the same preparation into the right knee-joint, the final intra-articular injection being 16 days before death. (H. and E. Magnification $\times 60$).

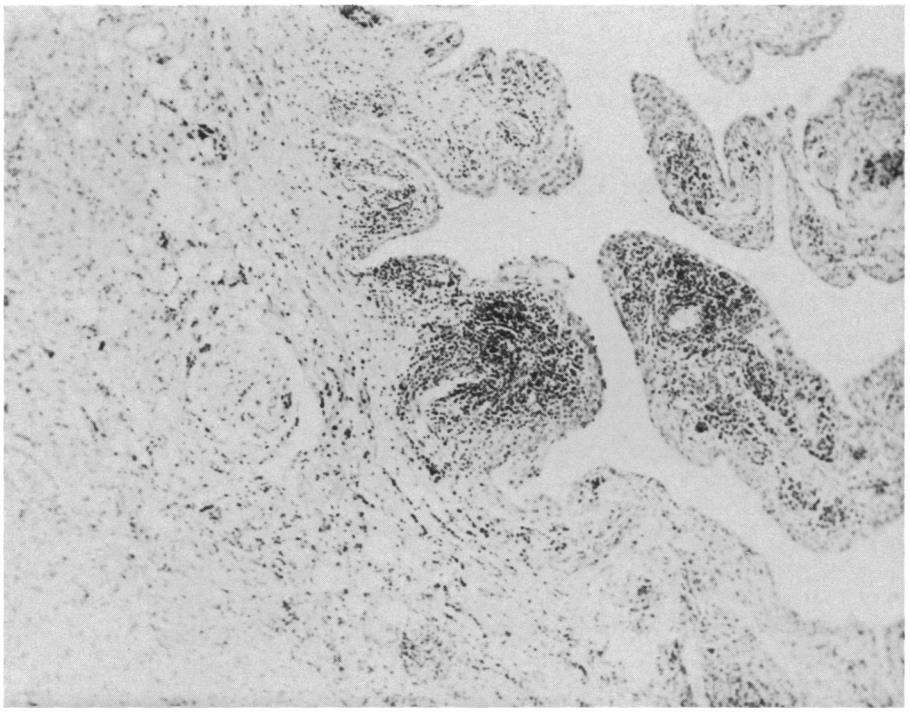

Figure 6. Inflammatory features in the synovial membrane of the left knee-joint of the same animal as described in Fig. 5, which followed immunization (asialylated IgG) with four intra-articular injections of saline. (H. and E. Magnification ×60).

DISCUSSION

The results presented describe an experimental rabbit model of arthritis which resembles the clinical condition of rheumatoid arthritis both histologically and immunologically. Asialylation of rabbit IgG by the enzyme neuraminidase (type V from *Clostridium perfringens*) rendered it alloantigenic in that an experimental rheumatoid factor-like antibody was produced. Intra-articular injections of asialylated IgG into presensitized rabbits induced swelling and severe inflammatory changes analogous to the joint lesions observed in rheumatoid arthritis. These findings are of particular interest as certain viruses are known to contain endogenous neuraminidase, e.g. *Haemophilus influenza*, which could possibly asialylate IgG *in vivo* and render it immunogenic. Proposals have already been made that viruses could be aetiological agent(s) responsible for inducing rheumatoid arthritis (Glynn, 1977; Vaughan, 1979).

Histological features characteristic of those seen in the joints of rheumatoid arthritis patients were apparent in the joints of the rabbits treated with asialylated homologous IgG. A chronic inflammation of the synovial membrane, of similar intensity to that observed by Dumonde & Glynn (1962), was observed in the infrapatellar fat pads of the asialylated IgG-injected knee-joints. This inflammation was characterized by a dense infiltration of mononuclear cells including plasma cells, with lymphoid aggregates frequently being observed. There was also evidence of similar, although much less severe, histological changes in the contralateral saline-injected knees of several of these rabbits, possibly suggesting the induction of a polyarthritis. The severity of the histological changes in the asialylated IgG-injected joints would suggest that the arthritis induced could be sustained by persistent, mononuclear-cell-mediated tissue destruction. Further studies to support this theory would involve examining the knee-joints of the animals at later periods of time after the intra-articular injections. Unexpectedly, the untreated IgG also proved capable of inducing mild inflammatory changes and tissue damage, when injected intra-articularly into rabbits

presensitized with the same IgG preparation. Although the reasons for this effect are unclear, it possibly reflects small changes which may occur during the isolation of IgG from pooled rabbit serum, or perhaps it demonstrates the expression of allotypic differences between rabbits.

The precise role of rheumatoid factor in the clinical disease is not clear, and is confused by the fact that many classical rheumatoid-arthritis patients are seronegative when tested for rheumatoid factor by the Rose-Waaler and latex fixation tests, whilst rheumatoid factors may be present in non-rheumatoid subjects with chronic infections, such as for example, subacute bacterial endocarditis (Glynn, 1977).

The results from this present study would tend to suggest that the presence of an experimental rheumatoid factor is not obligatory for the subsequent induction of an inflammatory response. The experimental evidence supporting this conclusion is threefold. First, immunization with asialylated homologous IgG was sufficient to promote the production of an antibody which was capable of agglutinating IgG-sensitized erythrocytes. This antibody is possibly directed against 'hidden' determinants on the asialylated IgG molecule, which become exposed by a charge-induced conformational change following asialylation. In this way the altered asialylated IgG might perhaps resemble that found in the human condition, where it is thought that in some way 'self' IgG becomes immunogenic and evokes the production of auto-antibodies, that is, rheumatoid factors (Milgrom, Dubiski & Wozniczko, 1956; Milgrom & Witebsky, 1960). However, there appeared to be no apparent correlation between the concentration of the circulating anti-globulin and the severity of the inflammatory response observed in the rabbits. Second, the animals immunized with untreated homologous IgG did not elicit an anti-globulin response, although other measurable variables proved this preparation capable of producing inflammatory and histological changes when injected intra-articularly. This lack of demonstrable anti-globulin activity may possibly be due to the immediate involvement of these antibodies in the formation of immune complexes, a hypothesis that is still much under review (Jasin & Cooke, 1978). Third, other investigators (Dumonde & Glynn, 1962; Consden *et al.*, 1971) have shown that an experimental arthritis can be induced using a protein antigen unrelated to IgG, such as ovalbumin or fibrin, although again no demonstrable anti-globulin was produced. Thus the experimental circulating rheumatoid factor is possibly just an interesting by-product of the arthritis, being produced in response to the aetiological agent used to induce the disease.

In conclusion it would appear that whilst it is possible to induce an arthritis in rabbits in whom there is no demonstrable anti-globulin activity, the prescence of this experimental rheumatoid factor in animals' circulation is not sufficient *per se* to initiate an inflammatory response. However, the subsequent intra-articular injection of antigen into a presensitized animal that already possesses circulating rheumatoid factor results in the induction of a much more chronic and persistent arthritis.

ACKNOWLEDGMENTS

We wish to thank Mr Ian Lewin for assistance with the animals used in this study. This work was supported by grants from the SRC and The Boots Company PLC.

REFERENCES

CONSDEN R., DOBLE A., GLYNN L.E. & NIND A.P. (1971) Production of a chronic arthritis with ovalbumin. Its retention in the rabbit knee joint. *Ann. Rheum. Dis.* **30,** 307.

DUC DODON M. & QUASH G.A. (1981) The antigenicity of asialylated IgG: its relationship to rheumatoid factor. *Immunology*, **42,** 401.

DUMONDE D.C. & GLYNN L.E. (1962) The production of arthritis in rabbits by an immunological reaction to fibrin. *Br. J. exp. Path.* **43,** 373.

GLYNN L.E. (1977) Recent concepts on the pathogenesis of rheumatoid arthritis. *La Ricerca clin. Lab.* **7,** 299.

HENNEY C.S. & STANWORTH D.R. (1965) The reactivity of rheumatoid factor with human gamma G globulin. *Immunology*, **9,** 139.

HUNNEYBALL I.M. & STANWORTH D.R. (1976) The effects of chemical modification on the antigenicity of human and rabbit immunoglobulin G. *Immunology*, **30,** 881.

JASIN H.E. & COOKE T.D. (1978) The inflammatory role of immune complexes trapped in joint collagenous tissues. *Clin. exp. Immunol.* **33,** 416.

MILGROM F., DUBISKI S. & WOZNICZKO G. (1956) Human sera with 'anti-antibody'. *Vox. Sang.* **1,** 172.

MILGROM F. & WITEBSKY E. (1960) Studies on the rheumatoid and related serum factors. 1. Autoimmunisation of rabbits with gamma globulin. *J. Am. med. Ass.* **174,** 56.

MURAYAMA J., TOMITA M., TSUJI A. & HAMADA A. (1976) Fluorimetric assay of sialic acids. *Analyt. Biochem.* **73,** 535.

STANWORTH D.R. (1960) A rapid method for preparing pure serum gamma globulin. *Nature (Lond.)*, **188,** 156.

STANWORTH D.R. (1966) Mechanisms of reactivity of rheumatoid factor with structurally altered and unaltered immunoglobulin. *Ann. Rheum. Dis.* **25,** 475.

STEWART G.A., HUNNEYBALL I.M. & STANWORTH D.R. (1975) The use of baboon IgG sensitized erythrocytes as an alternative indicator system in the study of interaction of rheumatoid factors with human IgG. *Immunochemistry*, **12,** 657.

VAUGHAN J.H. (1979) Rheumatoid arthritis, rheumatoid factor and the Epstein-Barr virus. *J. Rheumatol.* **6,** 381.