Perspective of antigen in nonarthritis joints

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Fox, A., and Glynn, L. E. (1975). Annals of the Rheumatic Diseases, 34, 431-437. Persistence of antigen in nonarthritis joints. The presence of antigen, IgG, and C3 was shown by radioautography and immunofluorescence in the collagenous tissues of the joints of animals injected intra-articularly with antigen after having been previously immunized with that antigen in Freund’s incomplete adjuvant. Since these joints were shown to be virtually free of inflammatory reactions, we suggest that the persistence of immune complexes activating complement cannot fully explain the chronicity of experimental allergic arthritis.

Experimental allergic arthritis, a chronic disease which histologically resembles rheumatoid arthritis was produced initially in rabbits by immunizing them with human fibrin in Freund’s complete adjuvant (FCA) and subsequently injecting the antigen intra-articularly (Dumonde and Glynn, 1962). It was soon realized that although Freund’s incomplete adjuvant (FIA) could not be substituted for FCA, as only an acute inflammation was produced, almost any soluble antigen could take the place of fibrin, and ovalbumin became the most commonly used antigen in this laboratory (Consden, Doble, Glynn, and Nind, 1971).

In experimental allergic arthritis, although most of the antigen disappears rapidly from the joint (Consden and others, 1971) and completely from the synovium (Doble, Dorling, Glynn, Webb, and Wilcox, 1973), some does remain localized in immune complexes in the surface layers of cartilage, ligaments, and menisci (Cooke, Hurd, Ziff, and JasIn, 1972a; Hollister and Mannik, 1974). C3 and immunoglobulin have also been shown in rheumatoid collagenous tissue (Cooke, Hurd, Bienenstock, JasIn, and Ziff, 1972b).

As an explanation for the chronicity of experimental allergic arthritis and indeed rheumatoid arthritis, it has therefore been suggested that persisting immune complexes activate complement which in turn causes the persisting inflammation. We present evidence that antigen injected into the joints of animals previously immunized with that antigen in Freund’s incomplete adjuvant also persists as immune complexes in collagenous tissues. Since these joints recover completely from the induced arthritis within a few weeks, we feel that the explanation for the chronicity of experimental allergic arthritis requires more than the mere presence of retained complexes.

Materials and methods

Materials
Rabbits of the Old English strain bred in our own animal house, and normal rabbit sera (NRS) and normal guinea pig sera (NGS) from our own bred animals were used. Ovalbumin was purchased from Koch-Light Ltd., Colnbrook, Bucks.; FIA from Difco Laboratories, Detroit, Michigan, U.S.A.; fluoresceinated sheep anti-rabbit immunoglobulin from Wellcome Reagents Ltd., Beckenham, Kent; fluoresceinated goat anti-rabbit C3 and anti-guinea pig immunoglobulin from Sera Services Ltd., Maidenhead, Berks. This latter conjugated serum was mixed with an equal volume of NRS, incubated for 30 minutes at 37°C, then centrifuged for 15 minutes at scale 10 on an MSE bench centrifuge, and the supernatant then removed and stored for use. Fluoresceinated rabbit anti-ovalbumin was prepared as follows. Sterile saline containing 20 mg/ml ovalbumin was emulsified with an equal amount of FIA. 1-0 ml was injected subcutaneously between the scapulae and this was repeated 2 weeks later. Four weeks later the rabbits were bled from an ear vein. The globulin fraction of these antisera was then separated and fluoresceinated (Johnson and Holborow, 1973). Guinea pig antiovalbumin was prepared as follows. Ovalbumin 20 mg/ml in sterile saline was emulsified with an equal amount of FIA containing 2 mg/ml Mycobacterium tuberculosis. One ml was injected into each animal. This was repeated 3 weeks later. Ten days later the animals were bled and the antiserum separated and stored at -20°C. [125I]Ovalbumin was prepared by preoxidizing with iodine, then radioiodinating by the Chloramine T method (Greenwood, Hunter, and Glover, 1963).
IMMUNIZATION METHOD
Ovalbumin, 20 mg/ml in sterile saline, was emulsified with an equal amount of FIA. One ml was injected into each rabbit subcutaneously between the scapulae. This was repeated 3 weeks later; another 2 weeks later 1.0 ml of sterile saline containing 12.75 mg of $^{125}$I ovalbumin was injected into the left knee joint. This dose contained approximately $1.8 \times 10^6$ cpm.

PROCESSING OF TISSUES
The animals were killed by injection of Nembutal in an ear vein. Both knee joints were completely removed. From each, one meniscus, a portion of intra-articular ligament, as much cartilage as could be shaved from both femoral condyles, a portion of synovium, and the popliteal lymph node were removed and placed in formol saline. They were counted in a Tracerlab gamma guard 150 and then processed for radioautography. The remainder of the joint was washed in several changes of phosphate buffered saline pH 7.3 (PBS) for about an hour. Then the 2nd meniscus, a portion of the cruciate ligament, the tibia, and attached patella from each joint was snap frozen in liquid nitrogen and processed for immunofluorescence.

RADIOAUTOGRAPHY AND HISTOLOGY
The formol saline-fixed tissues were embedded in paraffin wax. Several sections of each were cut at 7 $\mu$m. One was stained with haematoxylin and eosin (H&E). Two were radioautographed using a Kodak AR 10 stripping film with an exposure time of 2 and 4 weeks, respectively. After developing they were stained with haematoxylin.

IMMUNOFLUORESCENCE
Sections were cut at 6 $\mu$m and applied to gelatin-coated slides; sections of undecalciﬁed tibia, rather than cartilage sections, were cut on a Bright’s Free Standing Type Cryostat Model No. FSJS incorporating a Jung 11.50 rotary microtome, since cartilage sticks poorly to slides and attached bone helps adhesion. They were allowed to dry, then various conjugated antisera, diluted sufﬁciently to reduce nonspeciﬁc staining, were applied for 20–30 minutes. The slides were then washed for at least an hour in PBS. Guinea pig antiovalbumin was similarly applied, followed by washing for 15 minutes, then stained with conjugated anti-guinea pig immunoglobulin, absorbed as described above. Sections were mounted in one part glycerol, two parts PBS, and read within a few hours using a Reichert microscope equipped with a quartz-halogen light source, and dark ground condenser objective $\times 40$, n.a. 0.9; primary filter Balzer FITC 3, secondary filter Wratten 12. Photographs were taken using an Agfa CT18 film exposure time 20 seconds.

Sections from the un.injected right knee joint served as controls. In addition, since complexes were being looked for which if present would contain rabbit immunoglobulin, it was vital to show that the anti-guinea pig immunoglobulin was reacting with the guinea pig antiovalbumin applied first, and not with rabbit IgG endogenously present. Thus as an additional control the conjugated antisera were sometimes applied without previous treatment of the section and on other occasions by first applying NGS.

SKIN TESTS
Twenty $\mu$g ovalbumin in 0.1 ml sterile saline was injected intradermally, the skin having been depilated the previous day. The injection sites were observed at 4 and 24 hours.

HAEMAGGLUTINATION TITRES OF ANTI-OVALBUMIN
Performed as described by Zutshi, Reading, Epstein, Ansell, and Holborow (1969).

QUALITATIVE PRECIPITIN TESTS
Equal volumes of neat antisera and doubling dilutions of ovalbumin starting at 5 mg/ml down to 0.3 mg/ml were set up in capillary tubes. They were allowed to stand at room temperature and observed at 30 minutes and 24 hours.

Results

TISSUE RADIOAUTOGRAPHY
Portions of ligament, cartilage, and menisci from the left joint all showed substantial levels of radioactivity; pieces of synovium and popliteal lymph glands as well as all tissues from the right joint showed only background levels. The counts for one meniscus as a representative tissue are given in the Table. They were corrected for radioactive decay using the equation given and converted to $\mu$g of ovalbumin.

$$\ln \frac{A_t}{A_0} = 0.693 \frac{t}{T}$$

where $A_t =$ counts at time $t$

$A_0 =$ counts at time 0

$T =$ half life.

IDENTIFICATION OF ANTIGEN BY RADIOAUTOGRAPHY
Sections of meniscus, ligament, and cartilage from the left joint, but not synovium or popliteal lymph glands or any tissue from the right joint, showed clustering of silver granules just below the free surfaces (Figs 1–3).

Table Antigen retention in the menisci of left knee joints of rabbits each previously injected with 12.75 mg ($^{125}$I) ovalbumin, the animals having been first immunized with ovalbumin in FIA

<table>
<thead>
<tr>
<th>Time after injection (d)</th>
<th>Left meniscus (cpm)</th>
<th>Right meniscus (cpm)</th>
<th>Left meniscus minus right meniscus (cpm)</th>
<th>Left meniscus (corrected counts) (cpm)</th>
<th>Antigen (µg per meniscus)</th>
</tr>
</thead>
<tbody>
<tr>
<td>53</td>
<td>2249</td>
<td>155</td>
<td>2094</td>
<td>3862</td>
<td>0.27</td>
</tr>
<tr>
<td>56</td>
<td>5882</td>
<td>143</td>
<td>5739</td>
<td>10960</td>
<td>0.78</td>
</tr>
<tr>
<td>60</td>
<td>5200</td>
<td>158</td>
<td>5042</td>
<td>10082</td>
<td>0.71</td>
</tr>
<tr>
<td>63</td>
<td>3584</td>
<td>157</td>
<td>3427</td>
<td>7092</td>
<td>0.50</td>
</tr>
</tbody>
</table>
IDENTIFICATION OF ANTIGEN BY IMMUNOFLUORESCENCE

Indirect staining with guinea pig antiovalbumin followed by fluoresceinated anti-guinea pig immunoglobulin produced staining of the free surfaces of menisci, ligaments, and cartilage but not the synovia from the left joint or any tissue from the right joint. However, direct staining with conjugated rabbit antiovalbumin was consistently negative, despite the fact that two different preparations of fluoresceinated antiovalbumin were used and shown to give characteristic precipitin lines against ovalbumin in an Ouchterlony system. It was concluded that guinea pig antiovalbumin reacts at least in part with some sites different from those reacting with rabbit antiovalbumin, and these latter sites were already saturated with rabbit antiovalbumin (Fig. 4).

IDENTIFICATION OF IMMUNOGLOBULIN AND C3

The free surfaces of ligament, menisci, and cartilage, but not synovium, from the left joints or any tissue from the right joint were consistently stained with conjugated anti-rabbit IgG and anti-C3 (Figs 5–8).
FIG. 3 Radioautograph of articular cartilage from the right knee joint of a rabbit whose left knee was injected 56 days previously with 12-75 mg (125I) ovalbumin, the animal having been first immunized with ovalbumin in FIA. Note the absence of localization of silver grains. ×1250

FIG. 4 Ligament from the left knee joint of a rabbit injected 63 days previously with 12-75 mg (125I) ovalbumin, the animal having been first immunized with ovalbumin in FIA, stained with guinea pig antiovalbumin diluted 1:10, followed by fluoresceinated anti-guinea pig immunoglobulin diluted 1:16. Note surface staining. ×290

FIG. 5 Meniscus from the left knee joint of a rabbit injected 53 days previously with 12-75 mg (125I) ovalbumin, the animal having been first immunized with ovalbumin in FIA, stained with fluoresceinated anti-rabbit immunoglobulin diluted 1:32. Note surface staining. ×340

HISTOLOGY
The synovia from both left and right joints were examined after H&E staining and shown to be virtually free of inflammatory reactions.

SKIN TESTS
Four-hour reactions were very strong with oedema and erythema and had not diminished greatly at 24 hours. This is typical of a very strong Arthus reaction.

HAEMAGGLUTINATION TITRES
All were positive at 12800, the highest dilution tested.

QUALITATIVE PRECIPITIN TEST
All antisera were strongly positive in the capillary precipitin test.
FIG. 6 Ligament from the left knee joint of a rabbit injected 60 days previously with 12-75 mg (125I) ovalbumin, the animal having been first immunized with ovalbumin in FIA, stained with fluoresceinated anti-rabbit immunoglobulin diluted 1:32. Note the surface staining. ×360

FIG. 7 Articular cartilage from the left knee joint of a rabbit injected 53 days previously with 12-75 mg (125I) ovalbumin, the animal having been first immunized with ovalbumin in FIA, stained with fluoresceinated anti-rabbit immunoglobulin diluted 1:32. Note surface staining. ×360

FIG. 8 Ligament from the left knee joint of a rabbit injected 60 days previously with 12-75 mg (125I) ovalbumin, the animal having been first immunized with ovalbumin in FIA, stained with fluoresceinated anti-rabbit C3 diluted 1:20. Note the surface staining. ×340

Discussion

We have studied the persistence of antigen in the knee joints of animals previously immunized with ovalbumin in FIA and then intra-articularly challenged with ovalbumin. The presence of antigen in the surface layers of menisci, ligaments, and articular cartilage, but not in synovia or lymph glands, was shown by radioautography and immunofluorescence for periods up to 63 days, when the last of the animals was killed. In addition, the presence of rabbit immunoglobulin in washed joints was shown by immunofluorescence; immunoglobulin is known to be present in normal joint tissues but is easily removed by washing (Doble and others, 1973).

By radioactive counting the presence of substantial counts in ligament, cartilage, and meniscus, but not synovium or popliteal lymph glands, at periods ranging from 31–63 days was confirmed. In each meniscus alone counts equivalent to 0·27–0·78 μg of antigen were obtained. These figures are in the same range as those obtained by Cooke and others (1972a), where animals had previously been immunized with antigen in FCA. Consden and others (1971) found a rapid removal of antigen from whole joints being by far the fastest in normal joints, and substantially faster in animals previously immunized with antigen with FIA as compared to FCA. However, persisting antigen, after the first few days, was much the same whether FIA or FCA was used, namely 1·5 μg in each case, compared with about 0·2 μg in normal joints. Thus in the crucial period there is no obvious difference in the amount of antigen retained in the two groups of animals.

By skin tests and examination of joints at 24 hours it has been seen that the antibody produced in this system is capable of causing an Arthus reaction, and by implication is capable of fixing complement. This has been shown directly by immunofluorescent staining for C3. It has also been shown by haemagglutination and qualitative precipitin tests that circulating antibody is present in large amounts.

Thus immune complexes have been shown to persist in similar amounts in similar locations when antigen was injected intra-articularly whether the animal had first been immunized with that antigen in FIA or FCA. With the former, however, the animals do not develop chronic arthritis, whereas with the
latter they do. Thus the persistence of immune complexes activating complement cannot be the full explanation for the chronicity of experimental allergic arthritis. Indeed, in a more general sense our findings imply that the presence of immune complexes in a given disease state does not necessarily indicate that they have anything to do with that particular disease process. However, there are other possible explanations for the chronicity of experimental allergic arthritis.

The first explanation is that the slow release of antigen from the complexes sustains a continuing delayed hypersensitivity reaction in the joint of animals immunized with FCA. The need for this type of reaction to antigen, which is demonstrably not produced on immunization with FIA but requires FCA, has been stressed (Glynn, 1968). In addition, the production of a macrophage migration inhibitory factor by cultures of synovia from experimental allergic arthritis joints suggests a continuing delayed hypersensitivity reaction (Stastny, Cooke, and Ziff, 1973).

A second explanation is that at some stage during the initial inflammation autoimmunization occurs to some substance or substances in the inflamed joint. This could be involved in either a humoral or cell-mediated reaction or both. Evidence supporting this was obtained by Phillips, Kaklamanis, and Glynn (1966) who reported that inflammatory exudate (produced by subcutaneous injection of croton oil) injected in FCA, followed by intra-articular challenge with the same antigen some weeks later, causes the development of experimental allergic arthritis. In addition, leucocytes from adjuvant arthritic rats are inhibited by extracts of inflammatory tissue (Berry, Willoughby, and Giroud, 1973), while macrophages from mice having arthritis induced by *Mycoplasma pulmonis* are inhibited by extracts of normal synovial membranes (Harwick, Kalmanson, Fox, and Guze, 1973).

A third possibility is that during the acute phase, macrophages are attracted to the joint carrying with them the breakdown products of dead tubercle bacilli carried from the granuloma at the immunization site. Here they are released and may cause tissue damage in their own right or play the role of FCA in encouraging autoimmunization, in either case resulting in the further attraction of macrophages. It has been shown that the granuloma does persist as long as the arthritis (A. Doble and L. E. Glynn, unpublished observations). Moreover, the intra-articular presence of dead bacteria can cause an arthritis, e.g. dead streptococcal L forms (Cook, Fincham, and Lack, 1969), cell free extracts of *Erysipelothrix insidiosa* (White and Puls, 1969), and as little as 0.1 μg dead tubercle bacilli in FIA (A. Doble and L. E. Glynn, unpublished observations). Finally, macrophages carrying fluorescein group A streptococci pass to joints made arthritic by injection of streptolysin S (Ginsburg and Trost, 1971).

At this stage it is impossible to say which mechanism, or indeed combination of mechanisms, is at work in causing the chronicity of experimental allergic arthritis, but that something more than retained complexes alone is required.

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