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PERSISTENCE OF ANTIGEN IN RABBIT SYNOVIAL MEMBRANE

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SUMMARY.—It is already known that rabbits which show delayed-type hypersensitivity to an antigen will, after a single injection of the same antigen into a knee joint develop a chronic proliferative synovitis. It is also known that almost all of a foreign protein injected into a normal knee joint is rapidly cleared in a few days.

It has now been shown that if an animal is given foreign protein into a knee joint, and delayed-type hypersensitivity is produced later, that a chronic proliferative synovitis can also develop. This suggests that minute amounts of foreign protein can persist in an antigenic form in normal rabbit synovial membrane. It is possible that the persistence of this small amount of antigen may account in part for the chronicity of this form of experimental synovitis, and the fact that unlike human rheumatoid arthritis this type of experimental synovitis is confined to the joint injected with antigen.

DUMONDE AND GLYNN (1962) described a chronic inflammatory synovitis in the rabbit's knee joint resulting from an immunological reaction to fibrin. It was later shown that other foreign antigens could take the place of fibrin, provided the animal was immunized in a manner which produced delayed-type hypersensitivity to the antigen in question.

Glynn (1968) has speculated that the chronicity of this form of synovitis, which can last for at least 20 weeks, may be due to the development of a second immunological reaction to some product of the inflammatory process, rather than to the persistence of antigen. Using fluorescein and radio-iodine labelling of antigen, he had shown that ovalbumin as antigen disappeared very rapidly from the inflamed knee joint, as had previously been shown for normal joints, for example, by Rodnan and Maclachan (1960), who found almost complete disappearance of a variety of different proteins from animal joints around 3 days after injection.

We approached the problem of whether or not antigen might persist in a reactive form in synovial membrane by reversing the immunization sequence used by Dumonde and Glynn. The injection of antigen into the knee joint was given before the intracutaneous injection of antigen in complete Freund's adjuvant. The development of a chronic synovitis in these circumstances would we felt

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indicate the persistence of antigen in normal synovial membrane in reactive form but in very small amounts, at least up until the time when delayed-type hypersensitivity developed.

MATERIALS AND METHODS

Immunization.—Ovalbumin (Koch Light Laboratories Ltd: × 5 crystallized) dissolved in distilled water was mixed with equal parts of mineral oil (Difco incomplete adjuvant) containing finely ground dead dried human tubercle bacilli (Central Vetinary Laboratory, Weybridge, Surrey) so as to produce a water-in-oil emulsion with a final concentration of ovalbumin of 10 mg. per ml. and of tubercle bacilli of 1 mg. per ml. Immunization was by intradermal injection of 1·0 ml. of this emulsion in 5 or 6 sites between the scapulae.

Knee joint injections.—Under intravenous thiopentone anaesthesia, the skin around the knee joint was shaved and cleaned with 2% chlorhexidine in spirit. With the knee partly flexed the joint was entered either by a medial approach into the suprapatellar pouch or through the patellar ligament, when 10 mg. of ovalbumin in 1·0 ml. distilled water previously passed through a Millipore filter (0·22 μm.) was injected into 1 knee joint. One ml. of sterile saline solution was injected into the other knee joint.

Thirteen rabbits were given intra-articular ovalbumin into 1 knee joint and 1·0 ml. of saline into the other, and later immunized. The time intervals between the joint injection and the immunizing dose of ovalbumin are shown in the Table:—

TABLE

Number of rabbits	4	1	2	2	2	2
Time in days from joint injection to immunization	0	1	7	14	21	28

Four rabbits were given the joint injection on the same day as the immunization injection, and the longest interval between injections was 28 days. Six control animals were given intra-articular injections of ovalbumin and saline but not immunized.

Skin tests for the existence of delayed-type hypersensitivity.—An intradermal injection of 2 μg. of ovalbumin in 0·1 ml. water was made. The skin test was regarded as positive if 24 hr after injection there was an easily palpable area of induration at least 5 mm. across at the injection site. A positive skin test was taken to indicate the existence of delayed-type hypersensitivity to ovalbumin.

Histological examination of knee joints.—All animals were killed 4 weeks after the onset of delayed-type hypersensitivity and the 6 control animals 4 weeks after their joint injections. After dissection all joints were fixed in formol saline, decalcified and sections stained with haematoxylin and eosin were prepared. A sagittal section of the patella and tibia were made separately, as well as a coronal section through the femoral condyles.

Injection of ^{125}I labelled ovalbumin i.v. to look for uptake of ovalbumin by the knee joint and other tissues.—Ovalbumin was labelled with ^{125}I (carrier-free, from the Radio-chemical Centre, Amersham) following a modification of the method of McConahey and Dixon (1966).

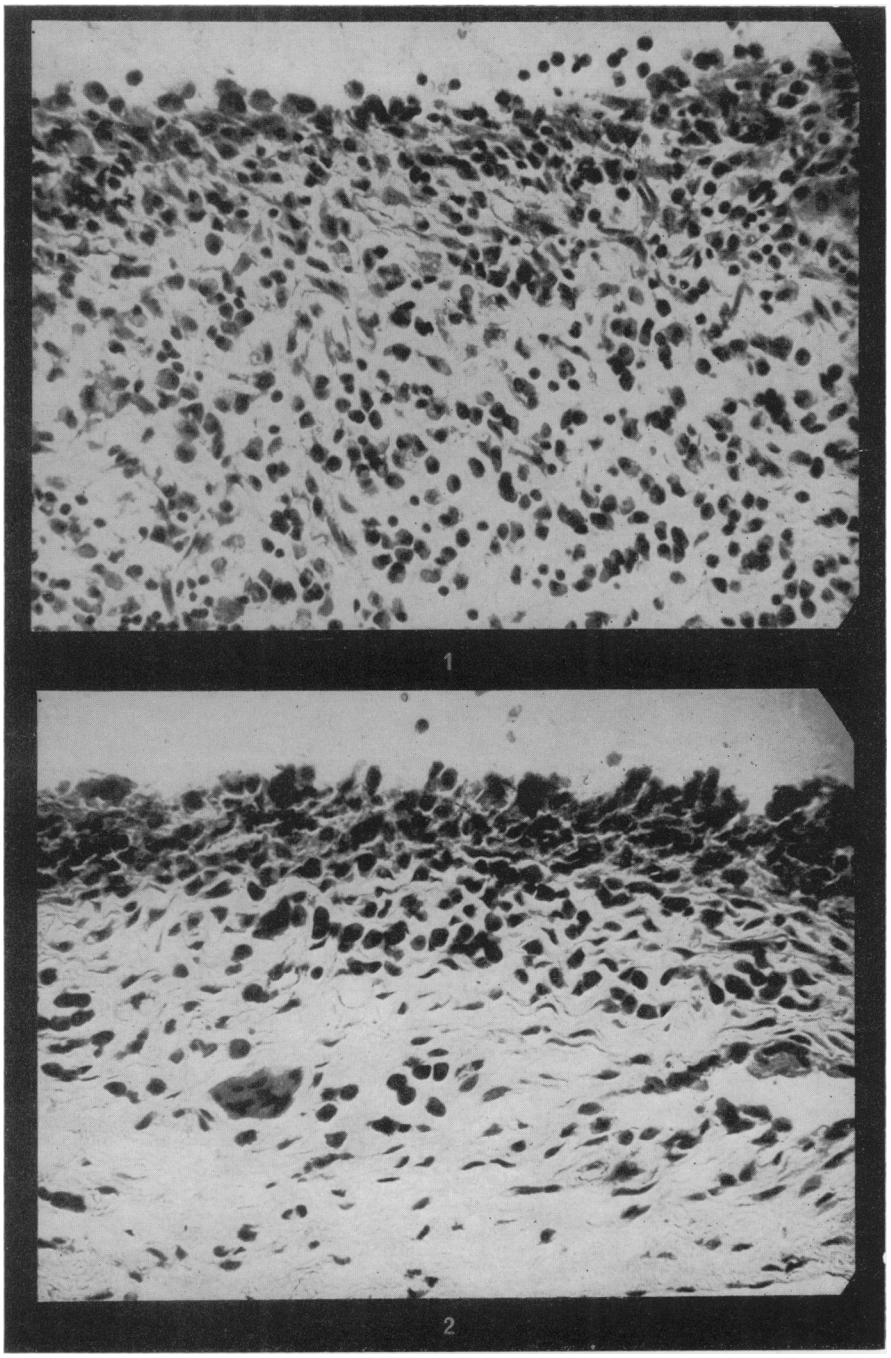
EXPLANATION OF PLATES

FIG. 1.—Coronal section of knee joint of a rabbit immunized with ovalbumin in complete Freund's adjuvant, on the same day as intra-articular injection of 10 mg. of ovalbumin in distilled water. Note the heavy infiltration of plasma cells and lymphocytes in the synovium. × 300.

FIG. 2.—Infiltration of hyperplastic synovium with plasma cells and lymphocytes in the knee joint of a rabbit given intra-articular ovalbumin 3 weeks before immunization with ovalbumin in complete Freund's adjuvant. × 300.

FIG. 3.—Coronal section of the knee joint of a rabbit immunized with ovalbumin in complete Freund's adjuvant 4 weeks after intra-articular ovalbumin. Note the hyperplasia of the synovial membrane with an aggregation of lymphocytes in the deeper part of the synovium. × 50.

FIG. 4.—Higher power view of the same field as Fig. 3 to show the edge of the lymphocytic aggregation, bordered by a zone of plasma cell infiltration. × 400.



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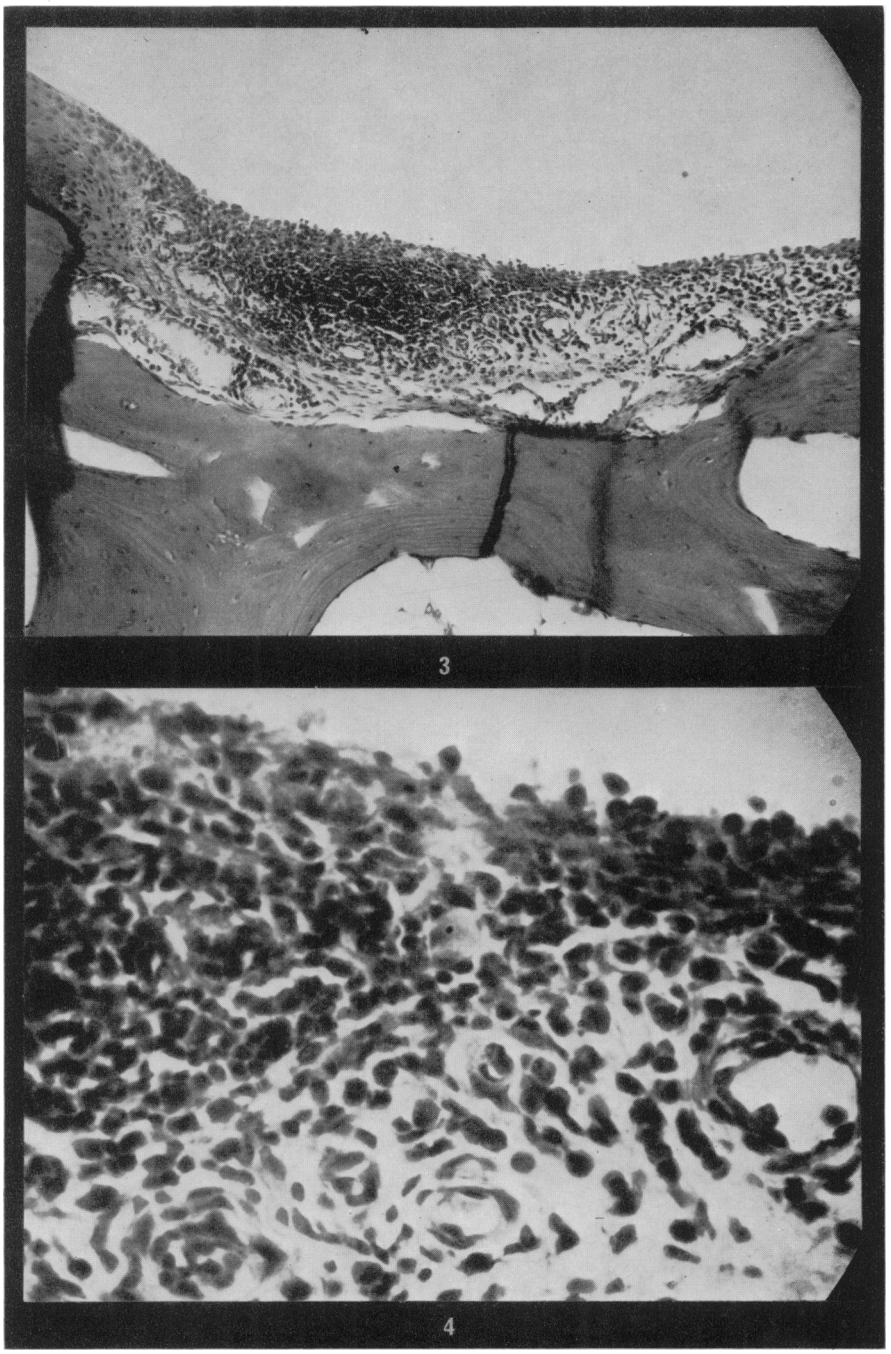

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A group of 3 rabbits were given intra-articular saline to one knee and intra-articular ovalbumin to the other, but not immunized with ovalbumin.

Fourteen days later each animal was given an intravenous injection containing approximately 10 mg. of ^{125}I labelled ovalbumin. At intervals of 2, 4 and 6 days after this intravenous injection, these 3 animals were killed, and various tissues were then counted in a well-type scintillation counter (Spectromatic; Tracerlab Ltd.). Samples of mesenteric fat, skeletal muscle, liver and spleen were taken, together with as much of the synovial membrane from each knee joint as could be obtained by careful dissection. After removal of the synovial membrane, the remainder of each knee joint was taken separately for counting.

RESULTS

All the immunized animals showed synovial membrane hyperplasia of the ovalbumin injected knee with a diffuse infiltration of the subsynovial layer with lymphocytes and plasma cells as seen in Fig. 1 and 2. The synovial layer of adipose tissue is seen to be separated from the synovial membrane by the inflammatory infiltrate.

Occasionally aggregates of lymphocytes were seen as shown in Fig. 3 and 4. No marginal erosions were seen, possibly because the synovitis was arrested after 4 weeks. None of the 6 control animals nor the saline injected knees of these 13 animals showed any inflammatory changes in the synovial membrane.

Two animals which were given intra-articular injections of saline and ovalbumin on the same day as the immunizing dose of ovalbumin were skin tested at daily intervals to make sure that delayed-type hypersensitivity did not develop until at least 4 days after the immunizing injection. One animal developed a positive skin test 11 days after immunization and the other 34 days after immunization. Because this second animal failed to develop a positive skin test 14 days after the first immunization a second immunizing injection was given, this being the only animal where this was necessary to produce delayed-type hypersensitivity to ovalbumin. All other animals gave a positive skin reaction when tested 14 days after a single immunization.

The severity of the inflammatory changes seen in the synovial membrane of the immunized animals varied, but there was no tendency for them to be less severe in those animals with the longer intervals between the joint injection and immunization.

In the group of 3 rabbits given intravenous ^{125}I labelled ovalbumin 14 days after ovalbumin had been injected into 1 knee and saline into the other, there was no evidence of any preferential uptake of ^{125}I by the synovial membrane, by the remainder of the knee joints (comparing the saline injected knee joint with the ovalbumin injected knee), nor by skeletal muscle or by mesenteric fat. These animals were killed at intervals of 2, 4 and 6 days after their intravenous injections. Only the liver and spleen showed any evidence of uptake of label.

DISCUSSION

The development of a synovitis lasting for several weeks in rabbits given intra-articular foreign protein at varying periods before immunization with the same protein, suggests that very small amounts of protein can persist locally in a reactive and possibly modified form for at least 4 weeks.

Although Rodnan and Maclachan (1960) showed that albumin and globulin from man and the rabbit were " virtually completely " absorbed from the rabbit

knee joint within 72 hr of injection, our results suggest that very small amounts of protein may persist for much longer.

It is possible that the mechanism of antigen persistence in the rabbit knee is similar to that described by Unanue, Cerotinni and Bedford (1969) for the persistence of antigen in peritoneal macrophages, for it is well known that a proportion of the synovial cells behave as macrophages.

The persistence of very small amounts of antigen on the surface of mouse peritoneal macrophages *in vitro* has recently been described by these workers. Using haemocyanins from the giant keyhole limpet (K.L.H. Molecular weight $7{\cdot}5 \times 10^6$) and from the spider crab they showed that small amounts of these antigens persisted on the macrophage surface after incubation at 37° C. for at least 72 hr. These workers estimated that an average of $1{\cdot}5 \times 10^4$ molecules of K.L.H. remained associated with single macrophages after a few hours incubation following an initial uptake of $7{\cdot}5 \times 10^6$ molecules.

In order to compare the amounts of antigen found to be retained by single macrophages *in vitro* by Unanue, Cerotinni and Bedford (1969), with rabbit synovial cells in the present work, it is necessary to make some estimate of the number of cells making up the synovial membrane, if it is known how much antigen is retained by the synovial membrane of the joint as a whole a few days after injection. The average area of synovial membrane which can be obtained by dissection from a normal rabbit knee joint is about 14 cm.^{2} (Webb, in preparation). If for this membrane a single layer of cells with a diameter of 25 μm. is assumed, then about $2{\cdot}9 \times 10^6$ cells are present at the membrane surface. Normal synovial membrane is often 2 or 3 cell layers thick, and although some of these cells are probably solely secretory in function, this estimate is probably on the side of too few cells. Recent observations using ovalbumin labelled with ^{125}I (Consden, Glynn and Nind, in preparation) have shown that after injecting 8·0 mg. into a normal rabbit's knee joint, there is a rapid clearance of the main part of the ovalbumin in the first few days, leaving at 7 days after injection 2·0 μg. in the entire joint and less than 1·0 μg. by the 28th day. Even at the earlier date, virtually all the persistent ovalbumin is found in the synovial membrane.

This amount of 1·0 μg. of ovalbumin (M.W., $4{\cdot}5 \times 10^4$) at 28 days, represents some $1{\cdot}34 \times 10^{13}$ molecules, so that something of the order of 4×10^5 molecules are being retained on average by each synovial membrane cell as previously estimated, giving an estimate a little over one order of magnitude of that found by Unanue, Cerotinni and Bedford (1969).

It was necessary to try to exclude the possibility that the injection of ovalbumin into the knee joint of a normal rabbit would lead to the formation of antibody to ovalbumin which would remain in the neighbourhood of the joint and there later bind ovalbumin escaping into the circulation from the immunization site. Such uptake into the joint would lead to an underestimation of the amount of ovalbumin persisting in the joint necessary to induce inflammation. However, no evidence could be found of ^{125}I labelled ovalbumin uptake by the knee joint synovial membrane in the 3 animals given intravenous ^{125}I labelled ovalbumin, where one knee joint had 14 days previously been injected with ovalbumin, and the other with saline for comparison.

It is also possible that naturally occurring cytophilic antibody to ovalbumin attached to the macrophage-type cell in the synovium would trap circulating ovalbumin. However, as all the saline injected knee joints were normal in animals

which showed a synovitis in the ovalbumin injected knee, if such cytophilic antibody is present, it is not able to trap antigen in significant amounts.

If synovial membrane can retain small but significant amounts of antigen for long periods, then it may be necessary to consider previous generalized infections as possible sources of antigen in diseases such as rheumatoid arthritis in man, where the clinical manifestations might be determined by changes in host reactivity to retained antigen.

Our findings suggest that it may simply be the persistence of a very small amount of antigen in synovial membrane which is responsible for the remarkable chronicity of this form of experimental synovitis, and they also provide an explanation for the development of a chronic synovitis only in those joints injected with antigen. This type of experimental synovitis, originally described by Dumonde and Glynn (1962), otherwise provides an excellent model for human rheumatoid disease.

More recent work (Glynn, in preparation) giving intra-articular ovalbumin in small amounts to rabbits previously immunized to ovalbumin has shown no significant inflammatory changes in the synovial membrane when 1 or even 10 μg. of ovalbumin is given into the joint. Only when 100 μg. was given into the joint was there obvious inflammation in the synovial membrane. This work contrasts with the results reported here, where as little as 1 μg. persisting in the synovial membrane before immunization has taken place, can lead to a significant inflammatory response. The antigen retained in the normal membrane would thus appear to have heightened antigenic properties, possibly by virtue of its position or form within or on the macrophages of the synovial membrane.

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