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Production of a chronic arthritis with ovalbumin

Its retention in the rabbit knee joint

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Dumonde and Glynn (1962) reported the production of a chronic arthritis in rabbits by the intra-articular injection of fibrin in animals previously immunized with this antigen in Freund's complete adjuvant. They commented particularly upon the persistence of the arthritis which was still active after 16 weeks. We report here further work, that with ovalbumin or bovine gamma-globulin as the antigen we can produce an arthritis which may still be active for 15 months, after a single intra-articular injection. Mr. B. Davis of Glaxo Laboratories Ltd. informs us that, in animals similarly treated, the arthritis has remained active for over 2 years. The mechanism underlying this persistence of the inflammatory process is obviously of great interest for the light it may shed on other chronic arthritides. It is, therefore, essential before invoking such debatable possibilities as auto-immunization, to determine both the amount of the initiating antigen remaining in the joint and the smallest amount of such antigen capable of exciting an inflammatory response. It was our purpose to obtain the necessary data to answer the question whether the persistence of antigen in the joint can alone account for the chronicity of the experimental arthritis.

Material and methods

The experimental animals were rabbits of the Old English strain bred in our own animal house. They were of either sex and weighed between 1,500 and 2,000 g. when first injected.

ANTIGENS

Ovalbumin was the thrice-crystallized material supplied by Koch-Light Co., Colnbrook, Bucks., and bovine gamma-globulin was the Fraction II of bovine plasma (Armour Pharmaceutical Co. Ltd., Eastbourne, England).

IMMUNIZATION

This was according to the following schedule. A solution of ovalbumin in 0·9 per cent. saline, 20 mg./ml., was emulsified with an equal volume of Freund's incomplete adjuvant (FIA) to which had been added 2 mg. dead, dried, well-ground human tubercle bacilli (kindly supplied by The Central Veterinary Laboratory, Weybridge, Surrey). 1 ml. of this emulsion was injected intradermally into the interscapular region at five sites, *i.e.* 0·2 ml. at each site. The injections were repeated 3 weeks later. After a further 10 days the animals were skin-tested by the intradermal injection of 20 μg. ovalbumin in 0·1 ml. saline. The results were read at 4 and 24 hrs to confirm the presence of both a humoral and a cellular response.

When animals were immunized with antigen in incomplete adjuvant, the tubercle bacilli were omitted from the emulsion.

IODINATION OF OVALBUMIN

^{125}I-labelling was carried out by the iodine chloride (ICl) technique of McFarlane (1958) and also by the chloramine T method of Hunter and Greenwood (1962), using ^{125}I-carrier free (Radiochemical Centre, Amersham). In both methods the total combined iodine amounted to about 1 atom/mol. protein. In the second method it was found necessary to use chloramine T in the proportion of 10 per cent. of the weight of ovalbumin. Before iodination with either method, the ovalbumin was pre-oxidized with iodine. A sample of this oxidized ovalbumin was obtained by freeze-drying a dialysed aqueous solution.

After iodination, unbound iodine was removed by dialysis against running tap water, then against several changes of cold physiological saline. The dialysed solution was centrifuged for 1 hr at 10,000 r.p.m. (Spinco) and the supernatant forced through a 'Millipore' filter (pore size, 0·22μ), to give a clear solution. The concentration of ovalbumin was calculated from its extinction at 280 mμ. assuming $E_{1cm.}^{1\%}$, 7·1 for ovalbumin. Over 95 per cent. of the total radioactivity was precipitated by trichloracetic acid. The concentration of the labelled ovalbumin was adjusted with sterile saline to about 8 mg. protein/ml. A sample of this solution was diluted with saline to give a suitable count rate and was used as a standard for counting.

Radioactivity was measured in a Tracerlab 'Spectro/matic' Scaler. Samples were placed in a well-type scintillation counter for counting. Solutions and specimens were counted in 100 × 15 mm. polystyrene tubes (Sterilin Ltd., Richmond, Surrey). The amount of labelled ovalbumin in the samples was determined by comparison

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of their counts with those of the standard.

Because the iodinated ovalbumin was pre-oxidized it was essential to know whether in this form it was able to elicit a chronic arthritis in rabbits immunized with the native unoxidized material. Preliminary experiments showed that the chronic arthritis obtained with the oxidized material was essentially identical to that obtained with the unoxidized.

ASSESSMENT OF ANTIGEN LEFT IN THE JOINT

The joint of the animal killed with an overdose of Nembutal was, except in Experiments 1 and 2, washed out by injecting 1 ml. saline and withdrawing the fluid after gentle massage of the joint. This was repeated three times in all. The joint was then opened and the synovial membrane was removed as completely as possible. The radioactivity in each of the washings and the synovium was determined in the counter and the amount of antigen present calculated, radioactive decay being allowed for by comparison with the activity remaining in the standard sample of the original material. In Experiment 4 (Table I), to avoid error due to antigen retained in parts of the joint not removed by dissection, the whole of the joint material (*i.e.* membrane, capsule, articular surfaces, and bone ends) was taken for counting.

HISTOLOGICAL EXAMINATION

Material for histology was fixed in buffered formol saline embedded in paraffin wax and sectioned at 5μ. Bone was decalcified by a formic acid formate method and for sectioning was double embedded in Celloidin in paraffin wax. All sections were stained with Ehrlich's haematoxylin and eosin.

ASSESSMENT OF ARTHRITIS

No attempt was made to assess the intensity of the inflammation during the first few days. Chronic arthritis was graded according to the density of the cellular infiltration and its extent. Four grades were recognized:

A trace ($\pm$) in which the synovium was normal but for an occasional plasma cell;

Slight (+) in which plasma cells and lymphocytes were sparsely scattered and mainly in the most favoured sites, namely the patella fringes, intercondylar fossae, and posterior joint space;

Moderate (++) in which the infiltrations are heavier, show a tendency to aggregate around small vessels, and are more widely distributed in the synovial membrane;

Severe (+++) in which the cellular infiltration is very dense and involves virtually all the synovium.

The three severer grades of inflammation as seen in the synovium around the patella are illustrated at low and high magnification in Figs 1 to 6.

Experiments and results

Before describing the results it is necessary to draw attention to three variables which must be taken into account before the different experiments can be compared.

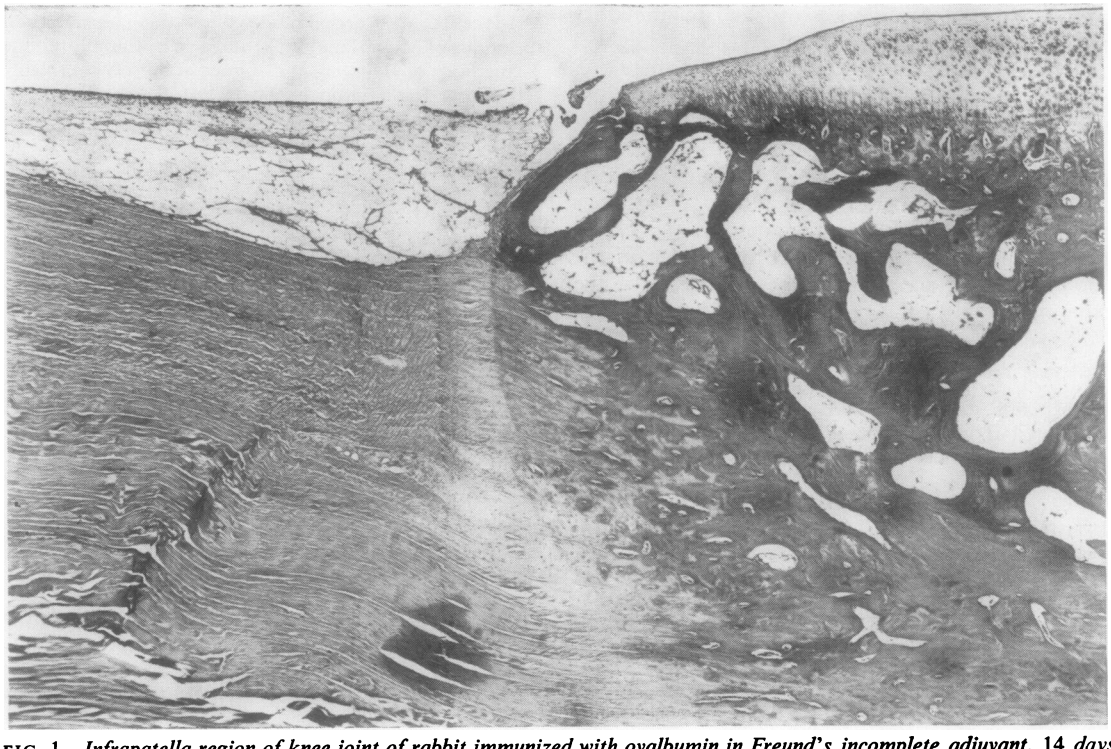

FIG. 1 *Infrapatella region of knee joint of rabbit immunized with ovalbumin in Freund's incomplete adjuvant,* 14 *days after the joint was injected with* ^{125}I *ovalbumin. Note slight villous hyperplasia and sparse cellular infiltration. Haematoxylin and eosin* × 30

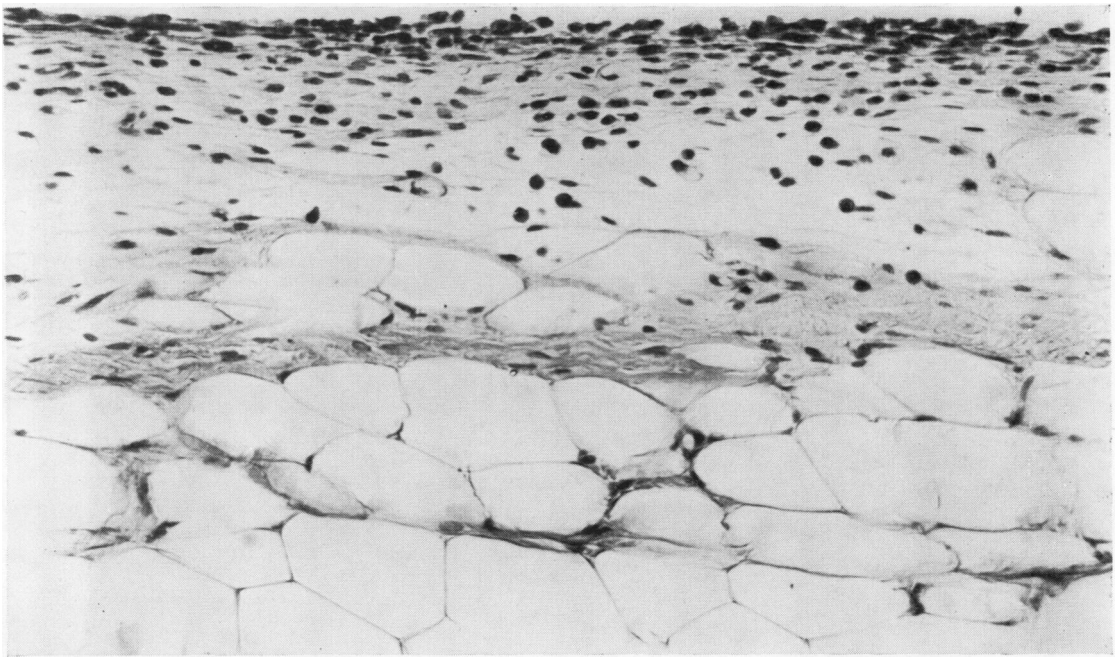

FIG. 2 *Higher magnification of synovial membrane of Fig.* 1, *showing scattered infiltration of lymphocytes and plasma cells. Haematoxylin and eosin.* × 300

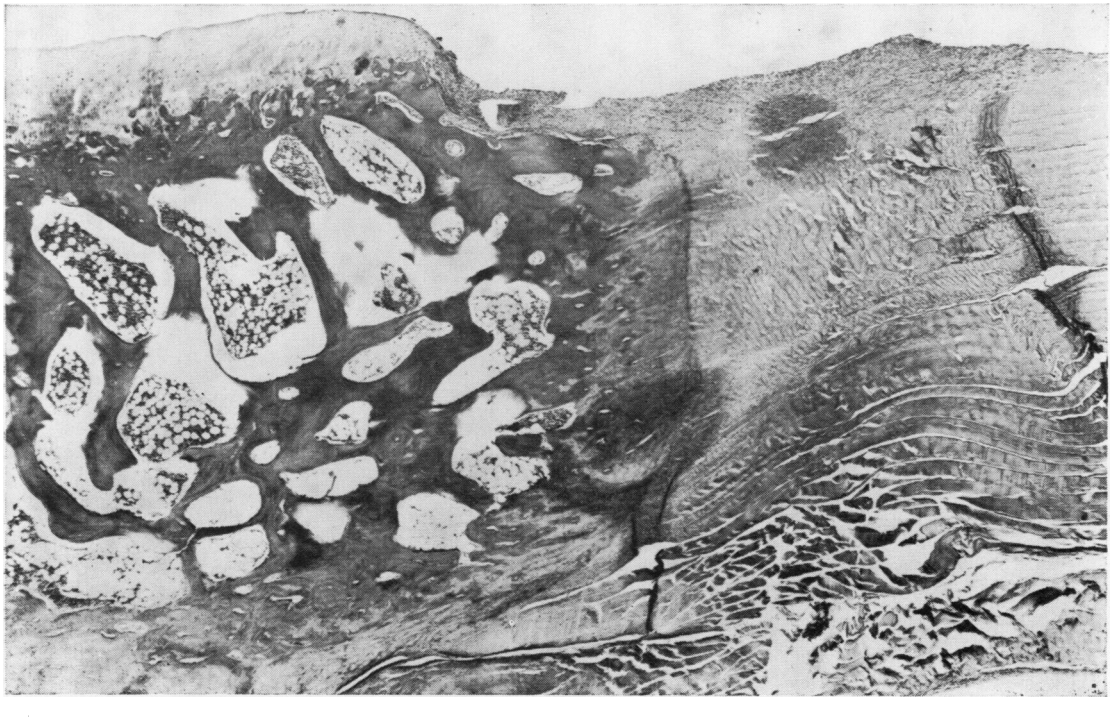

FIG. 3 *Suprapatella region of knee joint of rabbit immunized with ovalbumin in Freund's complete adjuvant,* 16 *weeks after the joint was injected with* ^{125}I *ovalbumin. Note moderate infiltration of cells in patella fringe with pannus formation and erosion. Haematoxylin and eosin.* × 30.

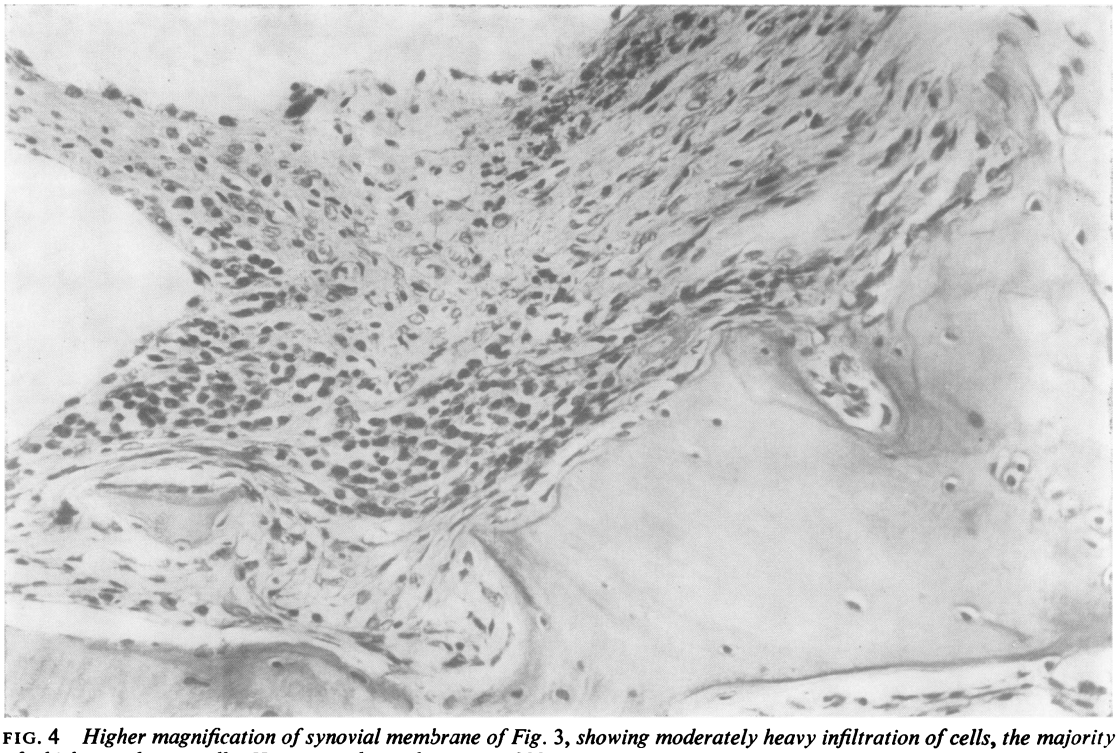

FIG. 4 *Higher magnification of synovial membrane of Fig.* 3, *showing moderately heavy infiltration of cells, the majority of which are plasma cells. Haematoxylin and eosin.* × 300.

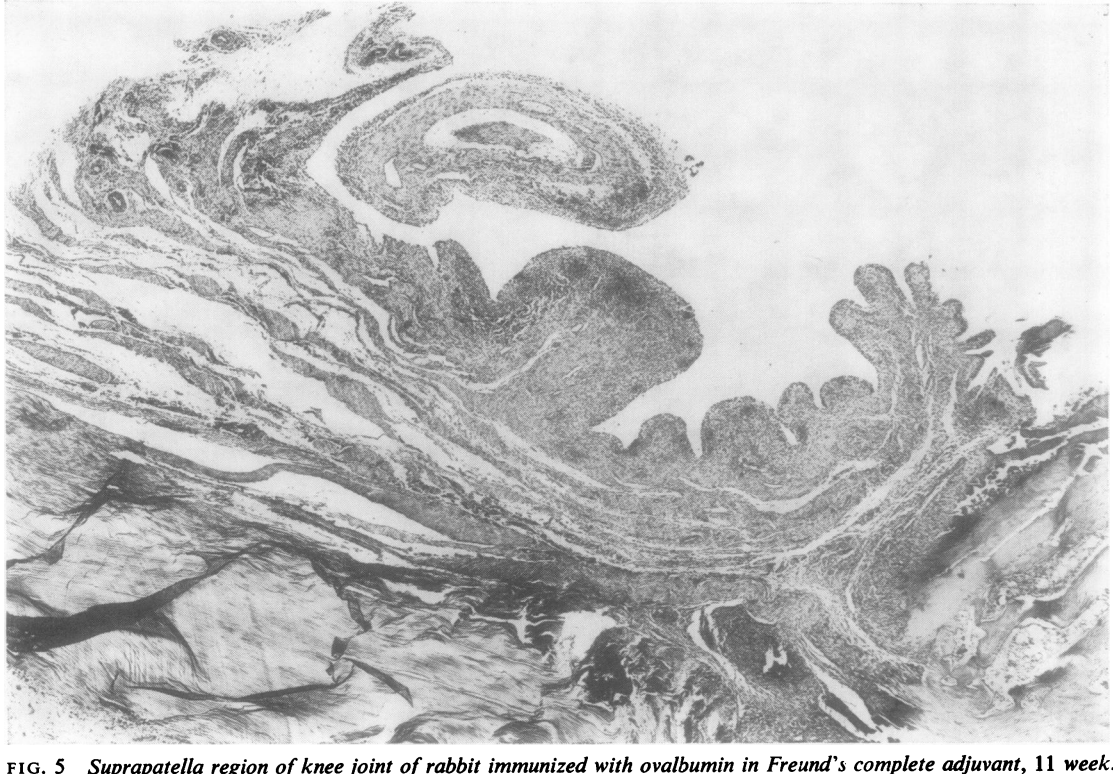

FIG. 5 *Suprapatella region of knee joint of rabbit immunized with ovalbumin in Freund's complete adjuvant,* 11 *weeks after the joint was injected with ovalbumin. The much thickened synovial membrane is heavily and diffusely infiltrated. Haematoxylin and eosin.* × 30.

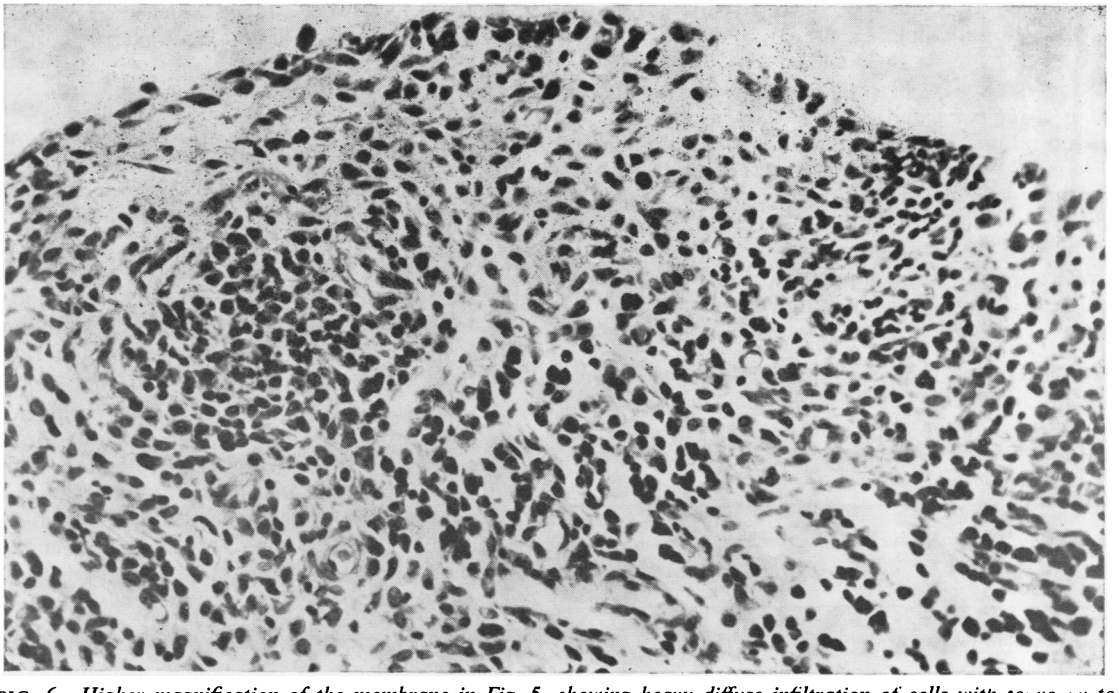

FIG. 6 *Higher magnification of the membrane in Fig. 5, showing heavy diffuse infiltration of cells with tendency to perivascular aggregation. Haematoxylin and eosin.* × 300.

(i) The ovalbumin iodinated with chloramine T was more resistant to removal than that iodinated with ICl (Table I). The difference was entirely lost by the 28th day.

(ii) In some experiments, the whole joint was taken for estimating the amount retained, whereas in others only the synovial membrane was taken. An analysis of the results with the former showed that the amount retained in the whole joint was approximately twice the amount retained in the readily removable synovium (Table I).

Table I *Comparison of antigen retained in whole joint with that present in synovium only*

Rabbit no.	*Iodination*	*Time after injection (days)*	*Antigen (μg.)*		
			In synovium	*In whole joint*	*Ratio**
206/6	Chlor. T	1	168·0	373·0	2·2
7		7	20·1	51·4	2·5
8		28	0·48	1·01	2·1
206/9	ICl	1	22·9	41·6	1·8
10		4	2·46	4·99	2·0
11		7	2·37	4·22	1·8
12		14	0·99	1·80	1·8
13		28	0·57	1·10	1·9
14		56			

* Quotient of $\frac{\text{Antigen in whole joint}}{\text{Antigen in synovium}}$

(iii) During the first few days a significant proportion of the retained material was present in the washings, but these were invariably negative by the 7th day.

EXPERIMENT 1 *Retention of ovalbumin in the knee joint of the unimmunized rabbit*

Five rabbits were used. Each was given 8 mg. (^{125}I) ovalbumin (ICl method) in 1·5 ml. saline into the left knee. One of these animals also received an injection into the right knee 18 hrs later and was killed after another 6 hrs, thus providing specimens after 6 and 24 hrs. The other four animals were killed on day 3, 7, 23, and 23. The antigen remaining is shown in Table II, from which it is evident that by 1 week less than 1 μg. remained even when the

Table II *Antigen remaining in knee joint of unimmunized rabbits after intra-articular injection of 8 mg. ^{125}I ovalbumin*

Rabbit no.	*Time after joint injection*	*Antigen in synovium* (μg.)*
197/11 R	6 hrs.	89·0
L	24 hrs.	14·0
12	65 hrs.	0·2
13	7 days	0·16
9	23 days	0·15
10	23 days	0·4

* The synovium was removed as completely as possible under direct vision.
The synovial fluid was not included for counting.

quantities found are doubled to allow for the incomplete sampling, *v.s.*

EXPERIMENT 2 *Retention of ovalbumin in the knee joint of the immunized rabbit*

Eight rabbits were immunized with ovalbumin in Freund's complete adjuvant and then challenged by an intra-articular injection of 8 mg. ^{125}I-labelled ovalbumin (ICl) and killed at the intervals shown in Table III. The residue of ovalbumin is obviously greater than in the unimmunized animals, some 100 or more days being required for the level of retained ovalbumin to fall below 1 μg. when the results are once again corrected for the incompleteness of the sample.

Table III *Antigen (ovalbumin) remaining in knee joint of rabbits immunized with ovalbumin in Freund's complete adjuvant*
Intra-articular injection consisted of 8 mg. ^{125}I ovalbumin in saline

Rabbit no.	*Time after joint injection (days)*	*Antigen in joint* (μg.)*	*Degree of inflammation*
197/1	28	8·5	+++
6	36	5·4	++
2	48	0·8	++
3	59	0·9	++
4	70	1·0	++
5	112	0·3	+
7	132	0·14	+++
8	132	0·7	+++

* The synovium was removed as completely as possible under direct vision.
The synovial fluid was not included for counting.

EXPERIMENT 3 *To compare the retention of ovalbumin in the joints of unimmunized rabbits to the retention in rabbits immunized with the same antigen in Freund's complete and incomplete adjuvants*

Immunization was as in Experiment 2, but with the omission of the tubercle bacilli from seven of the animals. Iodination was done by the chloramine T method. Each animal received 8 mg. ^{125}I ovalbumin and they were killed at the intervals shown in Table IV.

As the method of iodination had been changed, normal controls were included and killed at 24 and 96 hrs. It is evident that the early retention of the ovalbumin in these animals was considerably higher than in the corresponding controls in which the protein had been iodinated by the iodine monochloride method.

EXPERIMENT 4 *To compare the retention in the normal rabbit knee joint of ovalbumin iodinated by the ICl method to ovalbumin iodinated by means of chloramine T.*

Three rabbits were injected into the left knee joint with ^{125}I ovalbumin (chloramine T) 7·4 mg. in 1 ml. saline and six with ^{125}I ovalbumin (iodine monochloride) 8 mg. in 1 ml. saline. The animals were killed and the radioactivity in the washings and all

Table IV *Retention of antigen (OA) in knee joints of rabbits immunized with OA in complete (CFA) and incomplete (IFA) Freund's adjuvant*

Rabbit no.	*Immunization*	*Time after injection (days)*	*Antigen in joint* (μg.)*		*Degree of chronic inflammation*
			Inclusive	*Exclusive*	
16	CFA	1	978	694	
17		2	811	612	
18		4	562	476	
19		7	99	99	
20		14	1·1	1·1	++
21		27	4·7	4·7	+++
14		112	1·5	1·5	+++
15		476	n.d.	n.d.	+++
23	IFA	1	696	600	
24		2	111	99	
25		4	59	59	
26		7	3	3	++
27		14	1·4	1·4	+
28		27	1·6	1·6	+
29		71	n.d.	n.d.	+
31	None	1	196	123	
30		4	90	60	

* The inclusive figures include those for the washings. These were invariably free of labelled material by the end of the first week.

the components of the joint was measured. The results are presented in Table I.

It is evident from Tables I, II, and IV that for the first 7 days the retention of the antigen iodinated by the chloramine T method is considerably greater than the antigen iodinated with the aid of ICl. The reason for this is not known, but is probably due to a greater uptake of the former by the phagocytic cells of the synovial membrane.

The results in Table IV also show that, although for the first 7 days after injection there is considerably more retention of the label in the animals immunized with complete Freund's adjuvant than in those immunized with incomplete adjuvant, in both groups the retention is much higher at 24 hrs than in the non-immunized controls. The figures in the incomplete adjuvant group rapidly approach the control levels and are virtually identical by the 28th day, whereas in the CFA group retention is somewhat more prolonged. Thus at 28 days the control figure for the whole joint is 1 μg. (Table I, rabbits 8 and 13), whereas in the immunized animals (CFA group) the corresponding figures are 4·7 μg. (Table IV, rabbit 21) and 8·5 μg. (Table III, rabbit 1) for the synovium alone and are probably double this for the whole joint.

EXPERIMENT 5 *To determine whether the increased retention of antigen in the knee joint of immunized rabbits is specific or non-specific*

Ten rabbits were immunized as in Experiment 2, but with bovine gamma-globulin in place of ovalbumin. Freund's complete adjuvant was used. Each animal was then injected into the left knee joint with 10 mg. BGG and 8 mg. ^{125}I ovalbumin (chloramine T) in 1 ml. saline. They were killed and the radioactivity in the washings and synovial membrane determined as in Experiment 3. The results are given in Table V.

Table V *Retention of ^{125}I ovalbumin in knee joints of rabbits immunized with BGG and challenged intra-articularly with a mixture of BGG and ^{125}I OA*

Rabbit no.	*Time after joint injection (days)*	*Amount in joint* (μg.)*		*Degree of chronic inflammation*
		Inclusive	*Exclusive*	
200/1	1	899	692	
2	2	437	334	
3	4	423	388	
4	7	21	20	+++
5	14	0·5	0·5	++
6	27	0·36	0·36	++
7	97			+
9	475	—	—	+
10	474			+++

* The inclusive figures include those for the washings.

Comparisons of Tables IV and V shows that the early elimination of the labelled antigen is remarkably similar whether the immunization is to the labelled antigen or to some other unrelated antigen, but that after the first week retention is no greater than in the unimmunized controls (Table I). Apparently, retarded elimination is only specific after the first week which is presumably after the period of acute exudative inflammation, which itself seems to interfere with the normal processes of antigen removal.

EXPERIMENT 6 *To determine the retention of free iodide*

Six rabbits were injected into the left knee joint with 1 ml. of a solution containing 1μ.Ci of ^{125}I and 1 mg. cold sodium iodide. The animals were killed at 24 and 96 hrs and samples as shown in Table VI were removed for counting. By 96 hrs there was virtually no remaining radioactivity in the joint.

Table VI *Retention of ^{125}I when injected uncoupled into the rabbit knee joint*

Sample	*Corrected c.p.s.**	
	At 24 hrs	*At 96 hrs*
Standard 1 μ.Ci (1 ml.)	20,408	20,208
Washes (1)	0·2	0·0
(2)	0·0	0·0
(3)	0·0	0·0
Blood 1 ml.	1·65	0·0
Patella	1·03	0·0
Popliteal lymph node	0·0	0·0
Kidney (whole)	2·52	0·0
Knee joint including patella	3·28	0·17
Spleen	0·99	0·0
Liver	0·50	0·0

* Counts per second corrected for background.

EXPERIMENT 7 *To determine the minimal dose of ovalbumin necessary to produce a chronic arthritis still active 8 weeks after intra-articular challenge*

22 rabbits were immunized with ovalbumin in Freund's complete adjuvant as described above. They were then challenged with an injection of ovalbumin into the left knee joint with a dose ranging from 10 mg. to 1 μg. Two of the animals were killed at 4 weeks, and the remainder at 8 weeks (Table VII, overleaf). The results showed that a dose of 1 μg. or 10 μg. is insufficient to lead to a significant degree of arthritis: the maximum lesion at this dose range consisted of a very few widely scattered plasma cells. With a dose of 100 μg. or more almost all the rabbits showed a severe degree (Grade +++) of arthritis.

Table VII *Relationship between intra-articular dose of antigen and severity of the resulting arthritis*

Rabbit no.	*Dose of antigen*	*Time after challenge (wks)*	*Severity of arthritis*
3	10 mg.	8	+++
4	10mg.	8	+++
5	5 mg.	8	+++
6	5 mg.	8	+++
7	2 mg.	8	+++
8	2 mg.	8	+++
9	1 mg.	8	+++
10	1 mg.	8	+
11	1 mg.	8	+++
12	1 mg.	8	++
13	5 mg.	8	+++
14	5 mg.	8	±
15	100 μg.	8	+++
16	100 μg.	8	+++
17	10 μg.	4	+
18	10 μg.	8	±
19	10 μg.	8	±
20	10 μg.	8	0
21	1 μg.	8	±
22	1 μg.	4	0
23	1 μg.	8	±
24	1 μg.	8	±

All the animals had been immunized with the antigen (ovalbumin) in Freund's complete adjuvant. The arthritis was graded as described in the text.
± = normal synovium with an occasional plasma cell present.
0 = normal synovium.

Discussion

In accordance with the results of other investigators (Rodnan and Maclachlan, 1960; Hollingsworth, 1964), our findings confirm the rapid elimination of a foreign protein antigen from the normal rabbit knee joint. It is also apparent that for the first week at least there is a considerable difference in the amount retained depending upon the method used for iodination. The results with the unimmunized animals are brought together in Table I, which clearly shows that the ICl product is consistently better eliminated for at least the first week than the chloramine T product, but that difference is entirely lost by the 28th day.

It is probable that the difference in elimination rate is related to a difference in irritability between the two products, since a similar delay in elimination was shown when iodinated ovalbumin was injected into a joint inflamed by the simultaneous injection of another antigen, BGG, to which the animals had already been immunized (Table V). It is of interest that here too the abnormal retention of the marked antigen was only detectable for the first few days.

Since the difference between the two iodinated products in the normal joint is entirely lost by the 28th day, it is permissible to compare the retention of antigen after this period irrespective of the method of iodination used. Since, moreover, in the earlier experiments, retained antigen was determined in the easily-removed synovial membrane only, whereas in the later experiments the whole joint was taken, it is important to establish whether the relationship between the two quantities is sufficiently consistent to permit the application of a correction factor. In Table I the ratio of the antigen in the synovium to that in the whole joint is shown from which it is obvious that if the antigen in the synovium is multiplied by 2, a reasonably close approximation is obtained to the antigen in the whole joint.

A further difficulty in comparing the results of the early with the late experiments is the absence from the former of any data relating to the washings. The antigen in the washings, however, had invariably fallen to zero by the 7th day, so that only results before that time are affected, namely animals 197/11-12 in Table II.

All the results show that the elimination of antigen from the joint is a highly efficient process, that it is moderately delayed in the presence of acute inflammation and somewhat more delayed in the presence of specific immunization, but only when this is performed with Freund's complete adjuvant. In the absence of preimmunization the antigen retained in the joint 3 or more weeks after injection of about 8 mg. did not significantly exceed 1 μg. in any one of four animals (Table I, rabbits 8 and 13, Table II, rabbits 9 and 10) and may well have been much below this level, whereas in the animals challenged with the immunizing antigen this level was exceeded in seven of eight animals killed between days 27 and 112 (Table III, rabbits 1, 6, 2, 3, and 4; Table IV, rabbits 14 and 21), allowing again for the incompleteness of some of the samples.

The results of the experiment to determine the minimal dose of ovalbumin capable of exciting a chronic inflammatory reaction of at least 8 weeks' duration suggest that amounts of antigen under 10 μg. may be disregarded. Antigen retained in the joint may, however, be more effective than the same amount freshly injected, since the greater part of this latter is rapidly removed. Experiments to test this point (Ford, Webb, and Glynn, 1971) by delaying immunization for periods up to one month after the injection of antigen into the joint have indicated that the retained antigen is equivalent to 10–100 times the same amount freshly injected. That is, a quantity of 1 μg. retained antigen is equivalent to 10–100 μg. injected antigen and is capable of eliciting a chronic inflammatory response, which suggests that the inflammation in our experimental animals could well be maintained by persistent antigen for as long as this remains above 1 μg. Owing to loss of radioactivity it is not possible to

determine with any precision the time when the retained antigen falls below this critical level. From the shape of the retention curves (Fig. 7), however, it is extremely doubtful whether this time is ever much beyond 4 or 5 months. Some alternative mechanism must therefore be responsible for the lesions that remain active beyond this time.

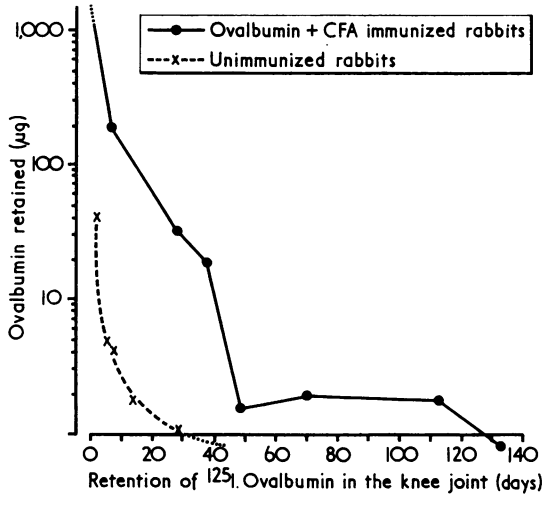

FIG. 7 *Curves of retention of* ^{125}I *ovalbumin in knee joints of rabbits after an intra-articular injection of* 8 *mg. Most of the points are single results but corrected where necessary for washings and residual tissues.*

Summary

The retention of iodinated ovalbumin (^{125}I OA) in the rabbit knee joint was estimated from the radioactivity remaining at various time intervals after its injection into the joint. Elimination from the normal joint was extremely rapid, about 1 μg. remaining 28 days after administration of 8 mg. Animals immunized with ovalbumin showed much greater retention during the first 4 weeks, but the amount retained fell to that retained by the unimmunized animal by about 8 weeks. By injecting ^{125}I OA together with bovine γ-globulin into the knee joints of rabbits immunized with bovine γ-globulin, it was found that retention in the immunized animal is partly the result of inflammation *per se* and partly specific.

The shape of the antigen retention curve suggests the possibility that retained antigen could be responsible for the persistence of inflammation for periods up to 6 months. It seems highly improbable that it could be responsible for inflammation lasting for more than 1 year.

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