Immunology, 1961, 4, 329.

Fluorescent Protein Tracer Studies in Allergic Reactions

I. THE FATE OF FLUORESCENT ANTIGEN IN ACTIVE AND PASSIVE ARTHUS REACTION IN THE GUINEA-PIG SKIN

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(*Received 28th September* 1960)

Summary. The fate of intradermally injected bovine serum albumin, labelled with lissamine rhodamine was studied in actively and passively sensitized guinea pigs, and compared with the fate of the same protein in control animals. In the latter, the antigen is taken up by macrophages, apart from a discharge of antigen to the regional lymph nodes.

In the sensitized animals, in which severe Arthus reactions occurred, an accumulation of antigen in vessel walls and lumina of capillaries and venules was observed. Polynuclear leucocytes take antigen after a few hours. This is a specific reaction absent with simultaneously injected protein to which the animal is not sensitized.

INTRODUCTION

The study of the fate of antigens and antibodies in allergic reactions is important from two points of view. In the first place such a study may throw light on the mechanism by which antigen-antibody reactions give rise to tissue changes. It is important to realize the the primary lesion caused by antigen-antibody interaction will occur at a molecular level, and probably will escape our attention when the common tracer techniques are used. But even in this case we can obtain indirect information about the site of these primary lesions.

Furthermore the study of the fate of antigen and antibody is part of the study of the pathogenesis of allergic reactions in general. It will give the opportunity to observe similarities and differences in the fate of antigen and antibodies in different allergic phenomena.

It was as part of this more general question that the study of the fate of antigen in active and passive Arthus reaction in the guinea-pig skin was undertaken.

MATERIAL AND METHODS

Animals. Male and female guinea pigs weighing ±250 g. were used throughout the experiments. A total number of eighty animals was used.

Antigens. Bovine serum albumin (Poviet, Amsterdam) labelled with lissamine rhodamine B200, according to Chadwick, Entegart and Nairn (1958) and the same protein labelled with fluoresceine isocyanate (Coons and Kaplan, 1950) and dimethyl naphthylamine sulfonylchloride (Mayersbach, 1958). Since in direct fluorescent tracing it is of

paramount importance to have a chemical conjugate only, without adsorbed dye, charcoal treatment was used (Chadwick and Nairn, 1960). With regard to impurities the conjugates were analysed by agar electrophoresis and sometimes by Ouchterlony technique. Control experiments were done with similarly labelled rabbit albumin, prepared from fresh rabbit serum (Kabat and Mayer, 1949).

IMMUNIZATION. *Active* immunization was obtained by intraperitoneal injection of bovine serum albumin 0.05 per cent 1 ml., mixed with 1 ml. Freund's adjuvant (Difco). A total of three weekly injections was given.

Passive immunization was obtained by intravenous injection of 2 ml. of a high titre rabbit antibovine-albumin serum (1:100.000).

INTRADERMAL INJECTION. Arthus reaction was provoked by intradermal injection of 0.1 ml. bovine serum albumin, 1.25 per cent, labelled with lissamine rhodamine or one of the other fluorescent dyes. In control experiments, Arthus reaction was provoked by injecting 0.1 ml. bovine serum albumin together with an equal amount of fluorescent rabbit albumin. In actively sensitized guinea pigs the injection was given 4 days after the last sensitizing injection. After passive sensibilization the Arthus reaction was provoked by antigen injection 24 hours after the sensitizing injection. Animals were killed $\frac{1}{2}$, 1, 2, 6, 18, 24 and 72 hours after the injection. At least four animals were killed on each occasion. Pieces of skin were fixed in neutral formalin or Carnoy mixture and embedded in paraffin. It was shown in preliminary experiments that after formalin fixation some of the extracellular protein diffuses from the pieces of tissue into the fixation fluid. This is not the case after Carnoy fixation which gives rise to an important disturbing auto-fluorescence and bad structural fixation. So the intracellular and precipitated protein is best studied in formalin-fixed material, the extracellular free protein after Carnoy fixation. Tissue sections were studied unstained by means of the fluorescence microscope and after staining with haematoxylin eosin by light microscopes.

RESULTS

THE FATE OF LOCALLY INJECTED BOVINE ALBUMIN IN THE SKIN OF NORMAL GUINEA PIGS

The intradermal injection of antigen in the quantities used in this experiment gives rise to a slight inflammatory reaction in the skin. There is an 'activation' of connective tissue cells; the cytoplasm of these cells becomes more abundant and basophilic in the haematoxylin-eosin stained sections. Furthermore there is hyperemia and leukodiapedesis, most marked in the loose connective tissue above the skin muscle. This acute inflammation is not very excessive, reaching its maximum within 24 hours. After the acute stage slight perivascular lymphocytic infiltration occurs, with occasional formation of tiny granulomas in the fat tissue after 24 and 48 hours (similar granulomas can also be seen after saline injection). The inflammatory reaction is caused by mechanical damage of the skin due to the injection, by disturbance of the colloid osmotic equilibrium and by the nature of the protein. So the elimination of protein after injection in a non-sensitized guinea-pig skin occurs in an area of mild inflammation.

Fluorescence Microscope Examination

Shortly after the injection of fluorescent protein there is a diffuse overall specific fluorescence of the skin in the region of the injection. No specific fluorescence can be seen

in the epidermis, hair follicles and skin muscle; they only show a bluish autofluorescence. The protein has penetrated into the extracellular tissue spaces and seems to be adsorbed on the collagen fibres. After a few hours there is a gradual fading of the overall fluorescence caused by a discharge of the protein by lymph into the lymph nodes. At the same time there is some uptake of protein in fixed connective-tissue cells. The number of protein-laden cells increases progressively and the protein becomes also visible in wandering cells (macrophages).

This process occurs throughout the dermis, but most extensively in the loose connective tissue above the skin muscle; in this region most of the inflammatory infiltrate can be found. Although the infiltrate is of a mixed cell type, especially after 24 hours, no uptake of fluorescent protein of any importance can be seen in granulocytes. This can be shown by comparison of photographs of the same spot in fluorescent and haematoxilin-stained sections. After 48 hours macrophages with fluorescent protein in their cytoplasm can only occasionally be found.

THE FATE OF LOCALLY INJECTED BOVINE SERUM ALBUMIN IN THE SKIN OF PASSIVELY SENSITIZED GUINEA PIGS

These animals all showed a severe Arthus reaction, macroscopically manifested by oedema and haemorrhage. The sections showed a marked infiltration with granulocytes, extravasation of red cells and thrombosis of vessels. The acute inflammatory reaction increased for 24 hours. After that time there was a progressive transformation from the granulocytic exudative inflammatory pattern into a more mononuclear proliferative one. The details of the histological appearance of the Arthus reaction provoked in our animals was in complete agreement with extensive studies by various authors (Gell and Hinde, 1953; Letterer, 1956; More and Movat, 1959). Later on some points which are important as to the fate of antigen will be considered in more detail.

Fluorescence Microscope Examination

Until shortly after the injection the slides from the sensitized animals showed the same fluorescent picture as those from the control animals. There was also a diffuse accumulation of protein in the extracellular spaces and adsorption of protein on collagen fibres. Part of the protein was discharged to the regional lymph nodes. But within half an hour after the injection there were differences compared with control animals, especially prominent at the edges of the injection spot. There were accumulations of antigen in capillaries and small veins, often the capillaries were nearly completely full of antigen. There was also an accumulation of antigen in vessel walls. In the walls of arterial vessels no antigen could be observed. The intravascular accumulations are partly in contact with endothelial cells, partly lying free within the lumen. In endothelial cells antigen could be clearly demonstrated (Figs. 1, 2, 3, 4).

Occasionally intravascular granulocytes could be observed with antigen in their cytoplasm (Fig. 7). In the tissue spaces, especially at the edges of the injection spot tiny particles of antigen could be observed.

Three hours after the injection the non-particulated overall fluorescence had decreased. The intravascular fluorescence was the same as before, as was the case with the accumulation in the vessel wall. From now on there were many extravascular granulocytes with intracytoplasmatic antigen (Figs. 5, 6, 7).

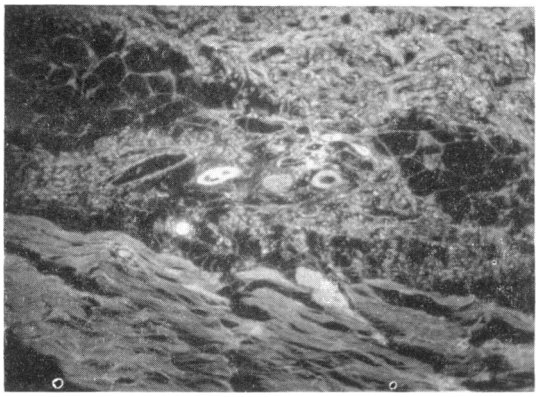

FIG. 1. Passive direct Arthus reaction ½ hour after provoking injection (fluorescence microscope picture, × 100). Fluorescence in vessel walls and intravascular fluorescence of bovine albumin labelled with lissamine rhodamine.

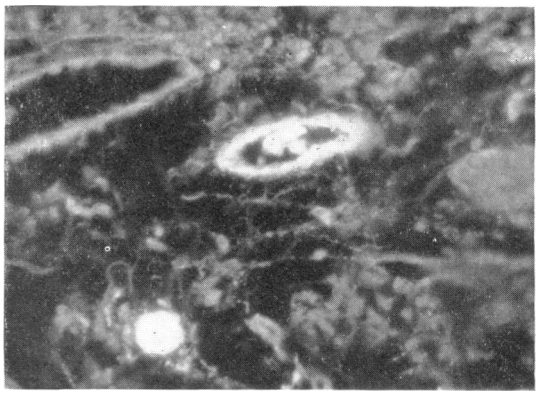

FIG. 2. Higher magnification of Fig. 1 (× 400.) Deposits of antigen in vessel wall and intravascular; extravascular precipitate in the connective tissue. One vessel obstructed by antigen deposits.

FIG. 3. Passive direct Arthus reaction ½ hour after provoking injection (fluorescence microscope picture, × 400). Accumulation of bovine albumin labelled with lissamine rhodamine in vessel wall.

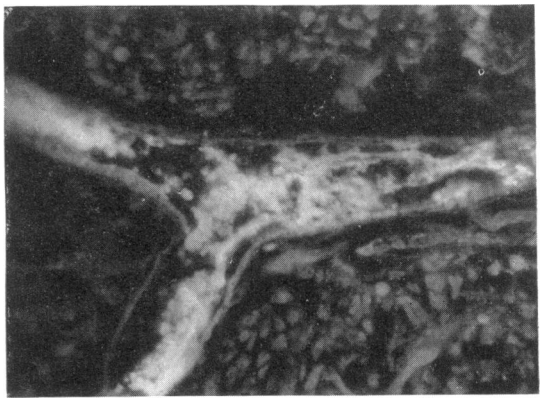

FIG. 4. Passive direct Arthus reaction ½ hour after provoking injection (fluorescence microscope picture, × 400). Intravascular deposits of bovine albumin labelled with lissamine rhodamine. Deposits of antigen in endothelial cells.

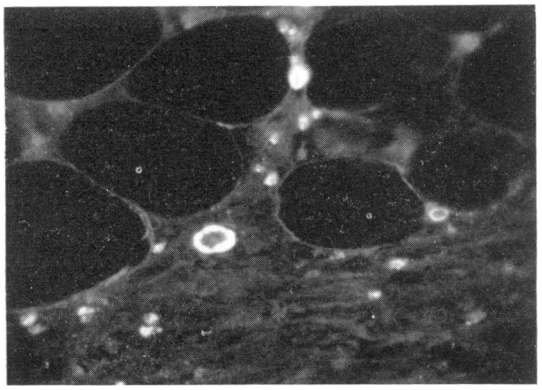

FIG. 5. Passive direct Arthus reaction 12 hours after provoking injection (fluorescence microscope picture, × 400). Deposits of labelled antigen in capillary walls. Uptake in granulocytes.

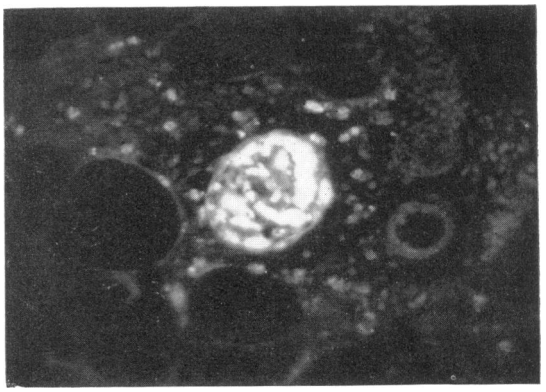

FIG. 6. Passive direct Arthus reaction 12 hours after provoking injection (fluorescence microscope picture, × 400). Intravascular accumulation of antigen. Some uptake of antigen in granulocytes in the inflammatory infiltrate around the vessel.

Some granulocytes had taken up lumps of antigen, others more tiny pieces. The granulocytes tended to accumulate around the extracellular particulated antigen and in vessel walls. During the next 18 hours there was a gradual disappearance of antigen from vessel

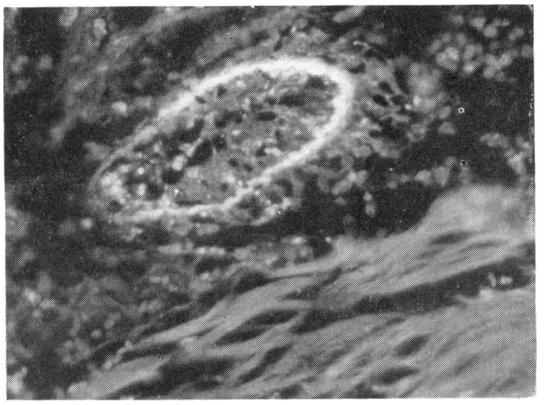

FIG. 7. Passive direct Arthus reaction 12 hours after provoking injection (fluorescence microscope picture, × 400). Deposits of lissamine rhodamine-labelled bovine serum albumin in venular wall. Intravascular and perivascular granulocytes showing uptake of labelled antigen.

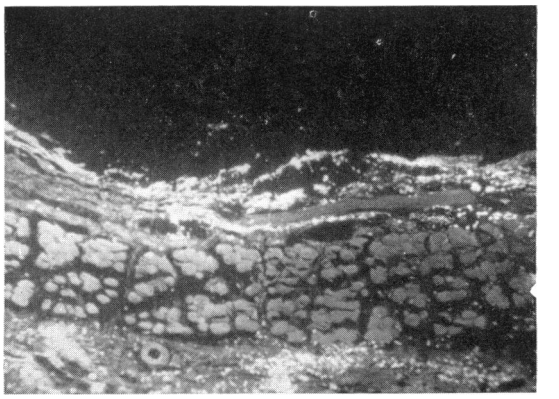

FIG. 8. Passive direct Arthus reaction 18 hours after provoking injection (fluorescence microscope picture, × 100). Accumulation of lissamine rhodamine-labelled bovine serum albumin in granulocytes inflammatory infiltrate around the skin muscle.

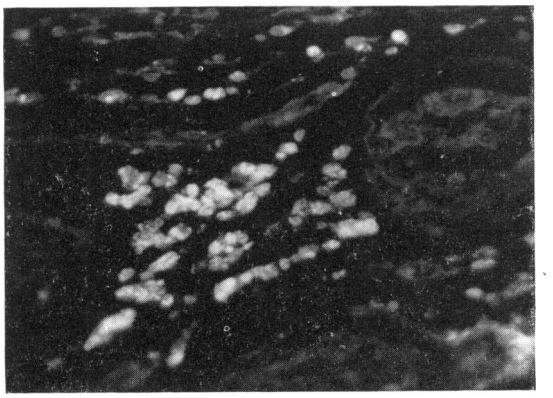

FIG. 9. Passive direct Arthus reaction 18 hours after provoking injection (fluorescence microscope picture, × 400). Uptake of lissamine rhodamine-labelled bovine serum albumin in granulocytes.

walls. More and more leucocytes were taking up the antigen (Figs. 8, 9). After that time there was a gradual disappearance from the leucocytes.

By photographic comparison of fluorescent and haematoxilin-stained slides it can be shown that macrophages and connective-tissue cells also take up the antigen. In the actively sensitized animals the fate of fluorescent bovine serum albumin was the same as in the passively sensitized animals.

CONTROL EXPERIMENTS IN WHICH ARTHUS REACTION IS PROVOKED BY SIMULTANEOUS INJECTION OF LABELLED RABBIT SERUM ALBUMIN AND NON-LABELLED BOVINE ALBUMIN

In order to study the specificity of these facts occurring with bovine serum albumin control experiments were undertaken.

In the first place it must be stressed that in experiments in which a mixture of labelled bovine albumin and non-labelled rabbit serum albumin is used to provoke a dermal reaction in guinea pigs, sensitized to the former, no difference could be observed in the fluorescence picture as described above. So the addition of rabbit albumin had no influence on the pattern of the reaction. In the sensitized animals injected with fluorescent rabbit albumin together with non-fluorescent bovine albumin (the specific antigen) a severe Arthus reaction occurred. The disappearance of the labelled rabbit albumin for the tissue was comparable to the disappearance in control animals. Only occasionally macrophages with fluorescent rabbit albumin in their cytoplasm could be observed. There was no uptake of fluorescent albumin by leucocytes.

DISCUSSION

In our experiments there is a clear difference in the fate of intradermally injected antigen in the normal and in the sensitized animal. In the normal there was a progressive disappearance from the tissue, the only real accumulation being intracellular in connective-tissue cells. In the sensitized animal there were accumulations of antigen in the lumina of blood vessels, in vessel walls and in the interstitial spaces of the skin. In both the antigen was discharged in an area of inflammation, granulocytes being present in the normal as well as in the sensitized animal. Only in the latter granulocytes were taking up the antigen. These differences were not due to the severity of the inflammation. When turpentine abscesses were provoked in guinea-pig skin, there was no uptake of fluorescent protein in granulocytes, neither after local injection of the labelled protein into the abscesses, nor when the animal carried intravenously injected protein in the circulation. Experiments concerned with provoking Arthus reactions by simultaneous injection of non-labelled bovine albumin and labelled rabbit albumin also stress the specificity of the phenomena in our experiments. There is uptake of the non-specific protein in connective-tissue cells, but no extracellular accumulations or uptake in granulocytes can be observed. There seems to be a retention of the protein to which the animal has been sensitized when compared with the non-specific protein. This is in contrast with the experiments of Korngold, Stahly, Dodd and Meyers (1953), who concluded from experiments with radioactive labelled protein that the retention was due to the non-specific inflammation. However, his observations were not on a histological level.

There are only a few and mainly contradictory papers dealing with the fate of antigen in Arthus reaction, studied on a histological level.

Waksman and Bocking (1953) studied the fate of intradermally injected ovalbumin and bovine gamma globulin in rabbits by means of the Coons technique. In Arthus reaction they found that only histiocytic cells were taking up the antigen; 'polymorphonuclear cells though present in considerable numbers are not found to contain intact antigen'.

More and Movat (1959) also think that the macrophage reaction represents the morphological counterpart of antigen removal in Arthus reaction. They consider that their

immunohistochemical investigations confirm their conclusion. Our results are in contrast with these findings and are in agreement with the findings of Cochrane and Weigle (1958) and Cochrane, Weigle and Dixon (1959). They studied the localization of antigen in rabbits by means of the Coons technique and could also find the deposits of antigen in vessel walls and the uptake of antigen by granulocytes. Their findings suggest that the antigen is taken up as a antigen-antibody complex. However, there are differences from our findings in guinea pigs. We could find accumulations of antigen in endothelial cells and considerable amounts of extracellular precipitated antigen, principally at the edges of the area of injection. Furthermore we want to stress the intravascular deposits of antigen, which are far more impressive, at least during the first few hours, than the accumulalations in the vessel walls.

If we try to describe the fate of the antigen in Arthus reactions more dynamically it seems to us that the following interpretation is nearest to what is actually happening.

Before the injection there is a high intravascular concentration of antibody. It seems plausible that there is at least some antibody in the extracellular spaces (Gitlin, Canding and Whipple, 1953; Ratzenhofer and Maresch, 1958). At the moment of the injection excess antigen is introduced into the extracellular spaces. So at first no precipitates but soluble complexes are formed extracellularly. It is possible that part of the primary interaction of antigen and antibody occurs in the vessel walls, but only in minute amounts. In the non-sensitized animal no antigen could ever be observed in vessel walls. Hence most of the primary interaction occurs in the extracellular spaces. This primary interaction of antigen and antibody probably gives rise to increase of vascular permeability and stases of blood in capillaries and venules. It has been shown that soluble complexes can produce increase of vascular permeability of the skin (Rosenberg, Chandler and Fischel, 1958).

This increase in vascular permeability causes a leakage of antibody into the tissues. Thus the ratio of antigen and antibody turns in favour of the latter, causing precipitation of antigen. It is clear that these extravascular precipitates will occur first at the borders of the injection spot, where the concentration of antigen will be lower than in the centre.

Owing to the injection there is an increase of the extravascular tissue pressure. This causes a back flow of tissue fluid into venules and capillaries at their venular sides. Especially in the vessels in which stasis occurs the haemodynamic pressure is very low. So the intravascular precipitates mainly occur in the prestatic and static capillaries and venules.

The further development of the lesion is due to the vascular damage and obstruction of the venules and capillaries on the venous side, causing a damage to the tissue, haemorrhage and more leakage of antibodies.

The granulocytes are attracted to the damaged tissue by the same stimulation as in other inflammatory reactions (Humphrey, 1959). Furthermore antigen-antibody complex is highly leucotaxic (Humphrey, 1959) at least in its insoluble form, the function of the leucocytes seems to be to clear away the insoluble precipitates. The present study once more stresses the importance of the granulocytes in Arthus reaction (cf. Humphrey, 1955; Cochrane *et al.*, 1959). It also clearly shows the possibilities of the use of direct labelled protein in the study of allergic inflammation. Further research on the correctness of the postulated fate of antibody in the Arthus reaction and the fate of antigen and antibody in other allergic reactions is in progress.

ACKNOWLEDGMENTS

This investigation was supported in part by a grant from the Organization for Health Research T.N.O. We wish to thank Dr. P. van Duijn (Biochemical section of our laboratory) for helpful advice and Miss J. C. Elskamp for technical assistance.

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