IDENTIFICATION OF SMALL QUANTITIES OF HUMORAL ANTIBODIES IN VIVO WITH THE AID OF LUMINESCENT ANTIGENS

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To detect small quantities of humoral antibodies in experiments on mice advantage was taken of the ability of an antiserum, if injected intradermally, to localize a fluorescein-labeled homologous antigen in the skin, the luminescence of which was observed in the luminescence microscope. The reaction is immunologically specific and is independent of the local increase in vascular permeability due to nonimmunological factors; it is more than 100 times more sensitive than the classic test of Ovary.

In experimental and clinical immunological investigations allergic skin tests are used, including the passive cutaneous anaphylaxis (PCA) test [4, 7]. Tests of this type are used not only to study the biological activity of a localized immune complex [5] but also to detect antibodies [7] and antigen [1]. The indicator of the formation of an immune complex is the local increase in vascular permeability at the site of intradermal injection of one of its components, and its intensity is estimated with the aid of an intravenously injected dye (Evans' blue) from the diameter of the blue stain on the inner surface of the skin. The guinea pig is considered to be the most sensitive animal on which to perform the PCA, but albino mice can also be used [2].

Although the PCA is more sensitive than methods of detecting antigen and antibodies in vitro, it does have one important disadvantage which is that nonspecific staining of the skin may occur through an increase in vascular permeability produced by nonimmunological factors (mechanical or toxic).

In the investigation described below the specificity and sensitivity of the PCA was studied when luminescence microscopy was used to evaluate the reaction.

EXPERIMENTAL METHOD

Noninbred albino mice of both sexes weighing 18-20 g were used. Two doses of antiserum were tested on each mouse and were injected intradermally into the lateral surface of the trunk in a volume of 0.1 ml. The homologous antigen was injected intravenously in doses of 1 and 4 mg. The antigen used was a commercial bovine serum albumin (BSA) conjugated with fluorescein isothiocyanate or rhodamine fluoride [3]. The protein content in the solution of fluorescent antigen was 16 mg/ml. The source of antibodies was rabbit immune serum against BSA with a titer of 1 : 320 in the complement fixation test (constant delay of hemolysis with a double dose of complement and with antigen in a concentration of 0.1 mg/ml). As a control of the specificity of the reaction, rabbit immune serum against Salmonella breslau was used. To per-

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TABLE 1. Sensitivity of the RAT Depending on Duration of Stay of Antiserum in Skin (4 mg antigen circulating for 24 h before reacting injection)

Dilution of serum	Duration of stay of serum in skin			
	30 min	3 h	24 h	24 h (control)
1:32	++++	++++	++++	—
1:64	++++	++++	++++	—
1:128	++++	++++	++++	—
1:256	+++	+++	++++	—
1:512	+	++	++++	—
1:1024	—	+	+++	—
1:2048	—	—	++	—
1:4096	—	—	++	—

form Ovary's test mice were sensitized intradermally with antiserum in different dilutions, and the antigen mixed with 0.25 ml 0.5% Evans' blue solution was injected intravenously 3 h later. In the reversed anaphylaxis test (RAT) an initial intravenous injection of antigen was given. The reacting injection of antiserum was given 1 or 24 h later, and this was followed after 10 min by an intravenous injection of the same dye. The reaction was read macroscopically by measuring the blue stain, and under the luminescence microscope by studying the intensity and area of spread of the specific luminescence in a section through the skin. The reactions were assessed 30 min and 3 and 24 h after the reacting injection.

Serial sections from the area of skin into which the serum was injected were cut on a cryostat at −25°C, fixed in 96% ethanol, and examined in the ML-2 luminescence microscope in short-wave blue light (FS-1-S3S7 entrance filters, ZhS-18 stop filter) with a dry 10× objective, aperture 0.4, and 40× water-immersion objective, aperture 0.65; and photographs were taken on RF-3 film using a 1.7× ocular. After examination, some sections were treated by the direct Coons' method with standard luminescent serum against rabbit globulins, prepared by the N. F. Gamaleya Institute of Epidemiology and Microbiology.

EXPERIMENTAL RESULTS

Preliminary experiments showed that when 1 and 4 mg BSA were used positive macroscopic results were obtained 30 min after the reacting injection in Ovary's test and in the RAT using antiserum in dilutions of not more than 1 : 32. In all experiments 24 h after the reacting injection the results were negative. A positive result in the luminescence-microscopic investigation was taken to be a greenish-yellow or orange-red luminescence (depending on the type of fluorochrome conjugated with the antigen) inside the vessels and also outside them in the surrounding connective tissue of the skin and subcutaneous tissue.

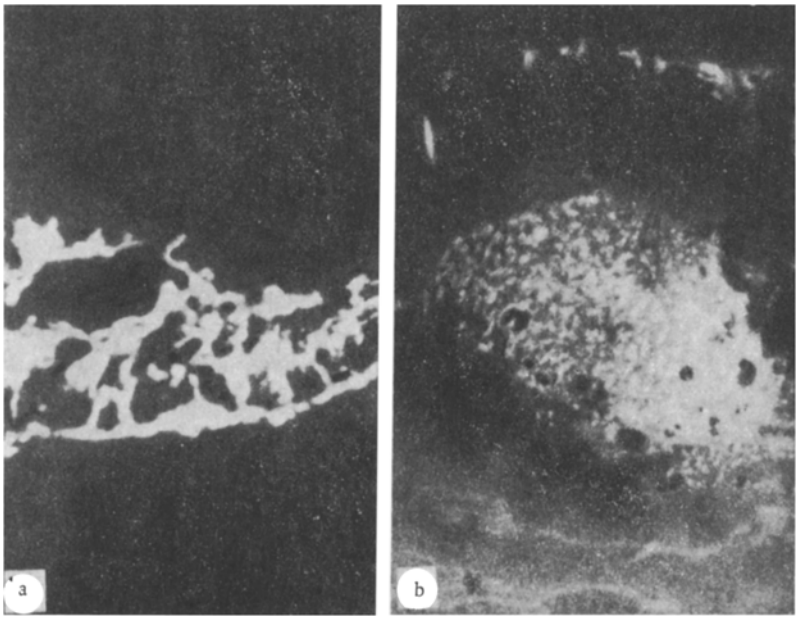

Fig. 1. Luminescence of antigen localized in the skin in the reversed anaphylaxis test (170×). Intravenous injection of 4 mg luminescent BSA, timed before reacting injection 24 h. Reaction at first as ++++ and +++ respectively 24 h after intradermal injection of antiserum diluted 1 : 64 (a) and 1 : 2048 (b).

The luminescent granules formed an immune complex, for after treatment of the sections by the direct Coons' method 24 h after intradermal injection a yellowish-green luminescence (antibodies) could be detected in those parts of the section where previously the orange-red luminescence of the localized BSA, labeled with rhodamine, had been found.

The extravascular localization of the precipitate was more marked in the RAT than in Ovary's test. This observation showed that luminescence microscopy could be used to detect minimal quantities of intradermally injected antibodies capable of combining in the RAT with antigen injected intravenously (BSA labeled with fluorescein isothiocyanate, which gives a brighter luminescence, was used).

The following scheme of evaluation was used: a widespread bright luminescence consisting of confluent foci was graded ++++ (Fig. 1a); well-marked luminescence in separate areas of the section, composed of a dense precipitate +++ (Fig. 1b); less dense focal luminescence of moderate brightness ++; scattered foci of weakly luminescent precipitate +.

The first series of experiments, performed on 24 mice, showed that the ability of antigen to become localized in the skin is increased if the time of its circulation in the blood is increased from 1 to 24 h before injection of the antiserum. Later, therefore, the ability of different dilutions of antiserum to localize the same dose of antigen was investigated, depending on the time of its stay in the skin (30 min, 3 and 24 h) after circulation of the antigen for the optimal time (24 h) before injection of the antibodies.

The results of this investigation, carried out on 72 mice, are given in Table 1.

As Table 1 shows, with an increase in the duration of stay of the antibodies in the skin the sensitivity of the RAT was considerably increased. Even in a dilution of 1:4096, the antiserum was still capable of localizing the antigen in a quantity sufficient to be recorded.

Besides high sensitivity, the modification of the PCA tested in these experiments also possessed marked specificity. By contrast with the classical Ovary's test, not even mechanical injury to the skin, produced by a metal clip, caused any localization of the luminescent material at the site of injury. Negative results also were obtained by the use of a heterologous system consisting of BSA and antiserum against Salmonella breslau.

In the analysis of the results it will be noted that the extravascular localization of the antigen was more marked in the RAT than in Ovary's test. The results indicating selective localization of the immune complex in places where antibodies are predominant [6] suggest that in Ovary's test antibodies injected intradermally before injection of the antigen were able to diffuse into the lumen of the vessel to a greater degree than in the RAT, when the antibodies interacted with antigen already fixed in the tissues.

The results are evidence that the ability of Evans' blue to pass through the wall of blood vessels is restricted to a comparatively short period during the development of the local allergic reaction.

This test of the ability of a serum to localize an intravenously injected homologous antigen, labeled with a fluorochrome, in the skin can be used as an extremely sensitive and highly specific modification of the PCA. The use of quantitative fluorometry, which is the writer's future intention, may make it possible to estimate the size of the resulting immune complex on a strictly quantitative basis.

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