THE EFFECT OF FREUND'S ADJUVANTS ON LYMPHATICS IN THE MOUSE'S EAR

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FREUND'S adjuvants have been used in many studies for the purpose of enhancing antibody production. The adjuvant mixture, in which the antigen is incorporated in a water-in-oil emulsion containing killed *Mycobacterium tuberculosis*, has also been described as having a stimulant effect on reticulo-endothelial tissue. A preparation containing tubercle bacillary wax was shown to produce widespread proliferation of plasma-cell elements in guinea-pigs (White, Coons and Connolly, 1955) and recently reticulo-endothelial changes with the adjuvant have been described in guinea-pigs, mice and Syrian hamsters (Laufer, Tal and Behar, 1959). It seemed possible that a further effect of the adjuvants might be to induce lymphatic proliferation, and the present study was undertaken to investigate this possibility.

MATERIAL AND METHODS

Thirty adult male albino mice were randomly allocated to 3 treatment groups A, B and C, which were given injections of saline, turpentine and Freund's adjuvant emulsion respectively. The mice were anaesthetized with ether and 0·005 ml. of the requisite fluid was injected subcutaneously in the centre of the right ear with an Agla micrometer syringe through a gauge 30 platinum-iridium needle. The left ear served as an uninjected control in each animal.

The adjuvant emulsion consisted of 2 volumes of saline, 1 volume of Arlacel A, 2 volumes of Bayol F and 1 mg. killed tubercle bacilli per ml. of emulsion. (Freund, Thomson, Hough, Sommer and Pisani, 1948.)

After injection the animals were left for 27–29 days and their ears were then examined for evidence of lymphatic proliferation. They were anaesthetized with pentobarbitone (6mg./ml., 0·4 ml. per 25 gm. body wt.), laid on a bed of plasticine, and their ears gently spread on wax moulds. The ears were observed with reflected light under a Zeiss binocular dissecting microscope at a magnification of × 10. To show up the lymphatics, Indian ink was injected through an Agla microinjection syringe with a quartz microneedle. The needle was introduced repeatedly into the skin and a very small amount of Indian ink injected each time, until it entered a lymphatic, when 0·005–0·02 ml. of Indian ink was injected.

For photography, a coverslip was mounted over the ear, and liquid paraffin was run into the space between the ear and the coverslip. An orange filter was used to increase contrast between blood vessels and lymphatics and exposures of 6 sec. were made on Pan F or FPa film with a 35-mm. camera. Both ears of each of the 30 mice were injected with Indian ink, observed, and photographed.

RESULTS

On examination of the surface of the ears with the naked eye and under magnification of 5× the following were observed :

Group A (saline).—In the 10 mice in this group no trace of the original injection site was seen. Six animals showed slight irregularity of the edge of their ears

or small scattered surface lesions on one or both ears, presumably due to scratching or fighting.

Group B (*turpentine*).—Lesions were noted in the right ears of all 10 mice in this group. These varied from mild oedema and congestion of vessels in the centre of the ear in 3 mice, to actual perforation or notching of the ear in 7 mice. The left ears were normal except for a few tiny surface lesions in 2 animals.

Group C (*Freund's adjuvants*).—In this group of 10 mice there was no obvious damage to the right ears. In most animals the site of injection could be seen on careful inspection as a slightly thickened area in the centre of the ear. There was no distortion of the vascular pattern and in only 1 mouse was there any congestion or increase of small blood vessels. Five mice showed slight irregularity of the edges of the ears. The left ears were normal except for irregularities of the edge of the ear in 2 mice.

On injection with Indian ink the following were observed :

Group A (*saline*).—The normal lymphatic pattern was seen in both test and control ears (Fig. 1), except in the case of 1 mouse in which the right ear showed patchy proliferation of lymphatics associated with some damage to the skin.

Group B (*turpentine*).—There was diffuse proliferation of lymphatics in the test ears as compared with the control ears (Fig. 2). This varied considerably from animal to animal, some showing a fairly dense meshwork of lymphatics and some showing only moderate proliferation.

Group C (*Freund's adjuvants*).—In 8 out of the 9 mice there was, in the test ear, a circumscribed area of very marked proliferation of lymphatics at the site of the original injection, showing up as a dense meshwork of vessels (Fig. 3). In the remaining mouse, only moderate proliferation at the site of the original injection was seen.

DISCUSSION

The effect of Freund's adjuvants on lymphatic proliferation has been compared with the effect of turpentine, chiefly because turpentine was one of the substances used by Pullinger and Florey (1937) in their classical studies on lymphatic proliferation. It is interesting to note that the proliferation of lymphatics in the turpentine-treated animals accompanied some degree of macroscopic damage of the ear, *i.e.* the proliferation was associated with either the healing of an ulcer or a perforation, or with an active inflammatory process, as evidenced by congestion and oedema. The proliferation of lymphatics in the mice treated with Freund's adjuvants, however, occurred without exception in the absence of marked macroscopic damage. The ears of these mice appeared to be normal on naked eye inspection, the thickening at the site of the original injection being obvious only on examination under the dissecting microscope.

The question of whether the lymphatic proliferation induced by Freund's adjuvant assists in the enhancement of antibody production is beyond the scope of this paper. The effect of adjuvants in potentiating antibody production is ascribed partly to retardation of the absorption and elimination of the antigen (Freund, 1947), partly to a general proliferation of plasma cell elements (White, Coons and Connolly, 1955) and partly to the production of a local granuloma containing antibody-producing cells. It is possible that a further factor may be proliferation of local lymphatics and thus preferential absorption of the antigen

into the lymphatic system rather than the general circulation. Gladstone and Abrahams (1958), discussing the possible significance of the local response in explaining the adjuvant effect, say " . . . since much of the antibody is formed in the regional lymph glands draining the site of injection, it might be argued that dispersal to lymph glands rather than local retension, was desirable." It is suggested that it might be worthwhile to investigate the effect of introducing adjuvants at a particular site a week or two before an immunizing injection, with the idea of preparing a " bed " of lymphatics in the area to receive the antigen. On the basis of the present experiments, however, one can go no further than to say that lymphatic proliferation occurs with Freund's adjuvants in the mouse's ear. It would be of interest to ascertain whether this proliferation is produced by one of the constituents of Freund's mixture or whether it is a function of their acting in combination.

SUMMARY

Lymphatic proliferation was studied in the ears of mice which had been injected with saline, turpentine or Freund's adjuvants a month previously. In mice injected with Freund's adjuvants, the ears showed little macroscopic damage but marked local proliferation of lymphatics at the site of injection. Mice injected with turpentine showed fairly extensive damage and varying degrees of lymphatic proliferation. Mice injected with saline showed no change from the normal pattern of lymphatics.

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EXPLANATION OF PLATE

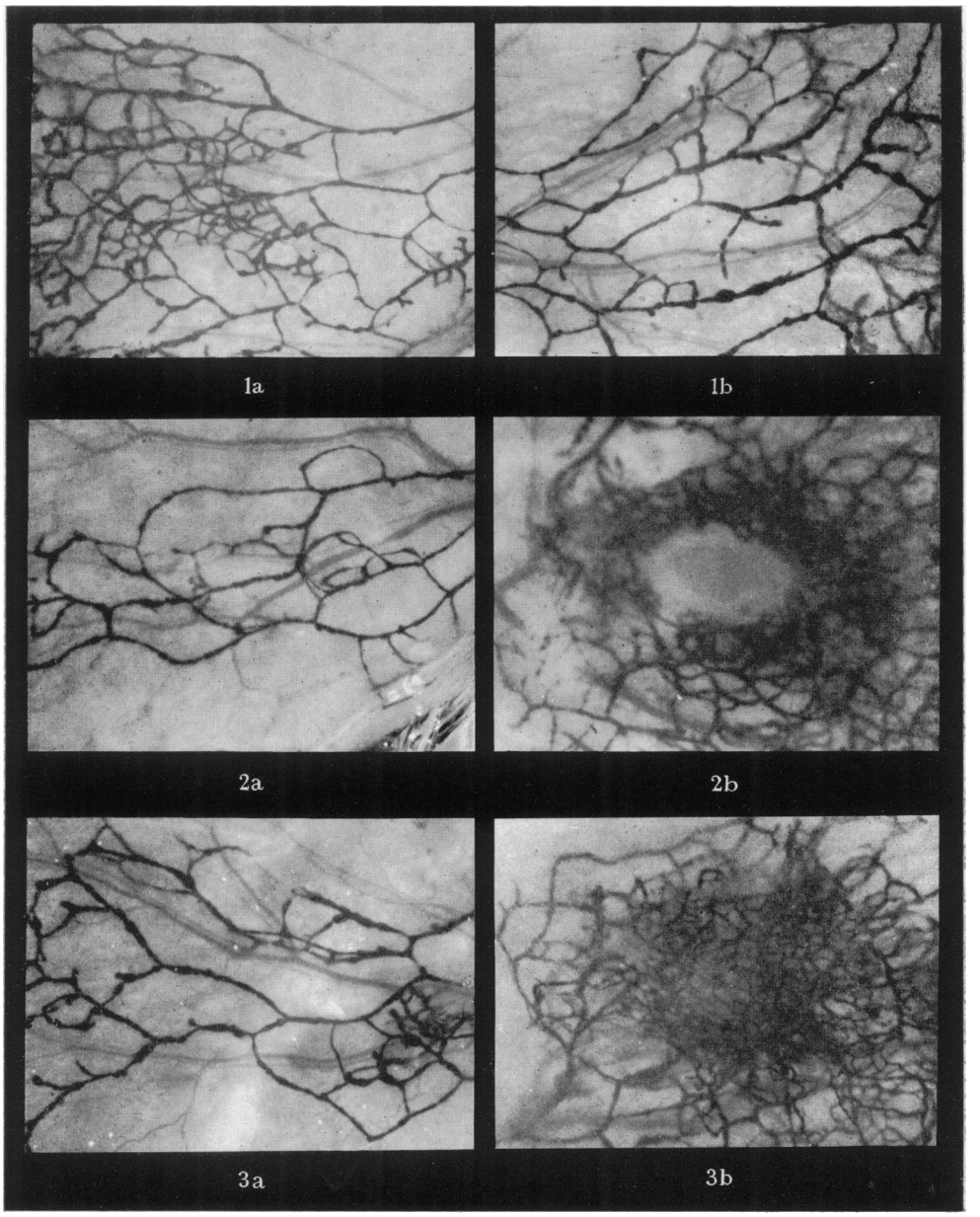
Photographs of mouse ears after injection with Indian ink, taken with reflected light at a magnification of 5 × using an orange filter.

(*a*) Left ears. (*b*) Right ears.

The right ears had been injected 29 days previously with saline (No. 1), turpentine (No. 2) or Freund's adjuvants (No. 3). The left ears were controls.

No. 2 (*b*) shows a perforation in the centre of the ear.

FIG. 1.—*a*—Control. *b*—Injected 29 days previously with saline.
FIG. 2.—*a*—Control. *b*—Injected 29 days previously with turpentine.
FIG. 3.—*a*—Control. *b*—Injected 29 days previously with Freund's adjuvants.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