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Delayed-Type Hypersensitivity in Mice Immunized with *Trypanosoma rhodesiense* Antigens

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When mice were immunized intravenously, subcutaneously, or by the footpad route with formaldehyde-killed *Trypanosoma rhodesiense*, delayed-type hypersensitivity was elicited by the use of frozen-thawed trypanosomal antigen. The delayed footpad swelling technique was used to measure delayed hypersensitivity. Hypersensitivity induction was dose dependent ($\geq 10^6$ formaldehyde-treated *T. rhodesiense*) and was affected by the route of immunization. The footpad route induced higher levels of hypersensitivity than other routes of immunization. Mice immunized with a single dose of formaldehyde-treated antigen and challenged with live *T. rhodesiense* did not survive. Yet, mice immunized subcutaneously with formaldehyde-treated antigen and then injected with frozen-thawed antigen and challenged 28 days after immunization survived. The results suggest that T-cell activation, manifested by delayed hypersensitivity responses, was a necessary component in the protective response, perhaps functioning in a helper cell capacity.

The study of the immune response to African trypanosomiasis has focused primarily on humoral responses (5). Characterization of the antibody response to trypomastigote infections, particularly to *Trypanosoma brucei* groups, has suggested that the parasites alter cell membrane antigenic determinants in response to antibody synthesis (10, 16). This shift in cell membrane antigens has led to the antigenic variation concept, which was proposed as a mechanism for parasite evasion and survival in the sensitized host. Additional studies showed that immunization with a cloned strain of trypanosomes, e.g., *T. brucei*, induced protection only against the immunizing clone, and not against other clones (3, 16). Further, protection was reported to be mediated primarily by B-cell and serum antibody (1, 12).

Cell-mediated immune responses in African trypanosomiasis have not been extensively studied. Delayed hypersensitivity (DH) reactions were observed in rabbits infected with either *T. brucei* or *T. rhodesiense* (15); yet DH could not be induced in rabbits immunized with trypanosomal antigens. Another study reported that DH could not be detected in rabbits infected with *T. congolense* (11). These contrasting results may reflect differences in host-parasite interactions.

Previously, it was reported that DH was detectable in mice immunized or infected with *Plasmodium berghei* (6, 7). The delayed footpad swelling (a T-cell function [8]) technique is used to assay DH reactions in mice (4). Thus, we decided to use this assay to determine whether DH could be induced in mice immunized with *T. rhodesiense* antigens—without the use of adjuvants. Further, immunized mice were challenged with live trypanosomes to assess whether DH could be correlated with protection. This report presents the results of these studies.

MATERIALS AND METHODS

Animals. Female, 8-week-old CD-1 mice (Charles River Breeding Laboratories, Wilmington, Mass.) and Fisher rats were used in these studies. They were given water and lab pellets ad lib.

Parasite. *Trypanosoma brucei rhodesiense*, Wellcome strain, was used throughout this study. The parasite was maintained in the laboratory by blood passage every 4 days.

Antigens. Mice and rats were inoculated intraperitoneally with 10^4 trypanosomes and exsanguinated when the parasitemia reached 10^8 to 10^9 parasites per ml of blood (4 to 5 days). The parasites were isolated by a method previously described (9) and then washed three times in phosphate-buffered glucose-saline (9) by centrifugation at 1,020 × *g* for 10 min at 10°C. The organisms were separated into two aliquots and treated as described below.

(i) Formaldehyde-treated (FO) antigen was prepared by suspending the washed trypanosomes in 10 volumes of cold, 0.1% formaldehyde-saline, pH 7.2, for 10 min, followed by centrifugation at 1,300 × *g* for 10 min at 5°C. The killed parasites were resuspended in

fresh, cold 0.1% formaldehyde-saline, counted in a hemocytometer, adjusted to 2×10^9/ml, and stored at 4°C until used.

(ii) Frozen-thawed (FT) antigen was prepared by adjusting the parasite concentration to 2×10^9/ml in phosphate-buffered glucose-saline. The trypanosomes were then rapidly frozen (−70°C) and thawed (30°C) four times. The FT antigen was stored at −70°C until used.

No live parasites from either antigen were detected either in wet preparations or upon injection into normal mice.

Immunization. Mice were immunized with FO antigen in a dose range of 10^3 to 10^8 killed organisms. The volumes used were 0.1 ml for animals immunized intravenously (i.v.) or subcutaneously (s.c.) and 0.05 ml for animals immunized in a hind footpad.

DH. DH was assayed by the delayed footpad swelling response and was elicited as previously described (6).

Challenge. Animals were challenged intraperitoneally with 10^3 trypanosomes per 0.1 ml. This dose caused death of normal mice in 5 days, with a fulminating parasitemia. Mice were observed for 30 days to assess protection.

RESULTS

Mice immunized i.v., s.c., or in a hind footpad with *T. rhodesiense* FO antigen became hypersensitive and developed DH when assayed with FT antigen (Fig. 1). Animals immunized with $\leq 10^5$ FO antigen showed minimal DH responses, whereas those immunized with $\geq 10^6$ antigen revealed DH responses twofold or greater.

Another set of experiments involved the kinetics of the hypersensitivity response in mice immunized as described above. Separate groups of mice were assayed for footpad responses 4, 14, 21, or 42 days after immunization. A minimum of 10 mice per dose per day were used, and no animal was tested twice. The results (Fig. 2) revealed that mice immunized i.v. or s.c. showed similar DH responses, and the peak occurred on day 4. Mice immunized in the hind footpad with

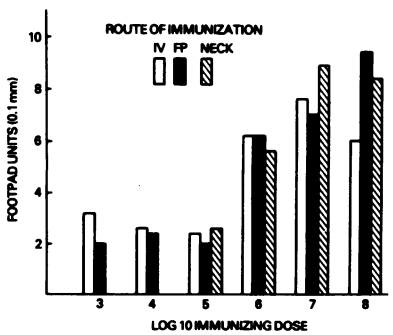

FIG. 1. *Effects of the route of immunization and dose of antigen upon the DH responses in CD-1 mice, measured on day 4. FP, Hind footpad.*

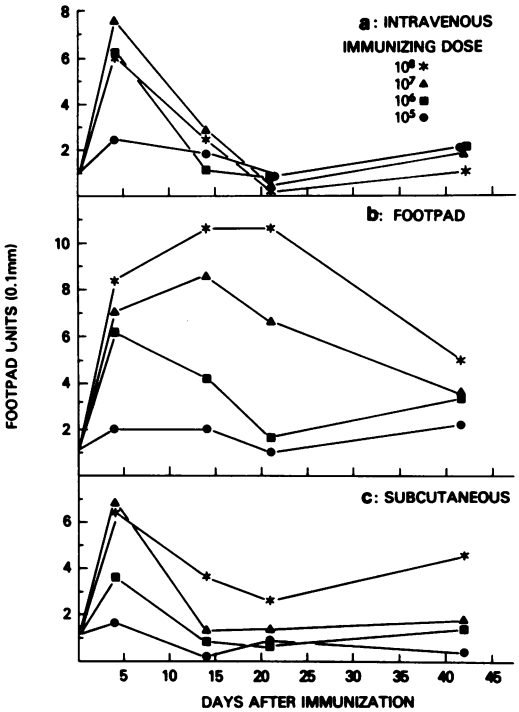

FIG. 2. *Effect of the route of immunization plus dose of antigen upon the kinetics of the DH responses in CD-1 mice.*

FO antigen revealed DH patterns that differed from mice immunized i.v. or s.c. (Fig. 2b). These differences were in the duration and peaks of hypersensitivity, which were directly related to the dosages of antigen used for immunization. A dose of 10^6 yielded hypersensitivity that peaked on day 4; the 10^7 group peaked on day 14, whereas hypersensitivity in animals immunized with 10^8 antigen plateaued on days 14 and 21 and then decreased by day 42. However, regardless of the route or dose of antigen, none of the mice survived when challenged intraperitoneally with 10^3 live trypanosomes.

Another group of mice were immunized s.c. with 10^5 to 10^8 FO antigen. They were then assayed for DH on day 4, 14, 21, or 42 after immunization. These mice were then challenged 14 days after they were assayed for DH (Table 1). The only protection observed was in mice that were assayed for DH on day 14 and challenged on day 28. Survival was directly related to both the immunizing dose of FO antigen and the time of FT antigen injection used for the DH assay (Table 1). A breakdown of the protected group (Table 2) revealed complete protection in those mice that were immunized, initially, with $\geq 10^6$ FO antigen, whereas the group

TABLE 1. *CD-1 mice immunized subcutaneously with T. rhodesiense antigen, assayed for their DH response, and then challenged intraperitoneally with 10^3 live T. rhodesiense*

Group[a]	No. survived/no. challenged
4/18	0/40
14/28	36/40
21/35	0/40
42/56	0/40

[a] Day (after immunization) of primary DH reaction (FT antigen injection)/day of challenge.

TABLE 2. *Influence of the immunizing dose and footpad injections of T. rhodesiense antigens, in CD-1 mice of the 14/28 group that survived an intraperitoneal challenge of 10^3 live T. rhodesiense*

Immunizing dose	Footpad dose	No. survived[a]/no. challenged
10^8	10^8	10/10
10^7	10^8	10/10
10^6	10^8	10/10
10^5	10^8	4–6/10

[a] Numerator gives range of mice that survived in two experiments.

immunized with 10^5 FO antigen were only partially protected.

DISCUSSION

These studies demonstrated that a delayed footpad response was elicited in mice immunized with *T. brucei rhodesiense* antigens without the use of adjuvants. The induction of a reproducible level of hypersensitivity was accomplished by the use of $\geq 10^6$ FO antigen. The route of immunization also affected the degree of hypersensitivity, and the footpad route was the most efficacious. The hind footpad site contained the antigen longer in situ (4) and provided a constant antigenic stimulation. By contrast, antigen injected i.v. or s.c. reached the tissues quickly and gave a rapid antigenic stimulation that resulted in a peak DH response on day 4.

The delayed responses we obtained were in contrast to results previously reported (15). The authors of that study were unable to induce delayed cutaneous reactions in rabbits with various *T. rhodesiense* antigens. The different procedures and animals used make direct comparisons difficult; yet the contrast between our and the previous studies point to the fact that the types of antigens, routes of immunization, and animals used affect the inducibility and/or detectability of DH in African trypanosomiasis. Another possibility is that DH may be detectable for only a short period of time in trypanosome-infected animals, as was previously reported in *P. berghei* infections in mice (6).

Regardless of the DH observed after a single immunizing dose of FO antigen, mice were not protected when challenged with live parasites. The data suggest that the hypersensitivity induced was to antigenic determinants on the FO antigens that either will not induce protection or will induce hypersensitivity but only an inadequate protective response. The latter concept is suggested by the data where protection was observed.

Mice that were protected received two doses of antigen: (i) FO immunizing antigen s.c. and (ii) FT antigen used for DH elicitation. These data indicate that the FO antigen primed the animals towards a protective response, and the injection of FT antigen completed this response. This effect was dose dependent (Table 2) because animals immunized with $\geq 10^6$ FO antigen and boosted with FT antigen were completely protected; the 10^5 animals were not. Complete protection was demonstrable only in mice that revealed a DH response of >2 units (>0.2 mm). Although the DH response was low at 14 days (Fig. 2c), when the mice were boosted, this does not obviate the possibility that T-cell activation was important, initially, in this system. We reported previously (6) that such a phenomenon was revealed in mice protected against lethal *P. berghei*; yet other studies have reported that B-cells and serum antibody were the factors responsible for protection in African trypanosomiasis (1, 12). The different antigens and immunization procedures make direct comparisons with our data difficult. Our data do not deny the role of B-cells and antibody in protection, but we argue that T-cells were necessary in our system to initiate the protective response.

Our data indicate that the FO antigen activated T-cells that functioned in DH and "memory" cells (2, 14) that were apparently short-lived. These cells were caused to further differentiate when the mice were assayed at the proper time (14 days) for DH. This may have resulted in the production of helper T-cells. Additional support for this concept is shown in Table 1, where mice that received FT antigen other than 14 days after immunization were not protected. Another possibility is that the FT antigen was a more efficient inducer, per se, of a protective response than was FO antigen; this is currently being studied.

The results reported here were found with CD-1 mice, which were outbred animals. It is known that mice that vary at the H-2 locus respond differently to the same antigens (13). Whether other strains of mice will reveal similar hypersensitivity and/or protection needs further

study. This is particularly needed with inbred strains, so that adoptive cell transfer can be accomplished. This will aid in defining the roles of B- and T-cells in the protective immune response to *T. brucei rhodesiense*.

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