INFECTION AND IMMUNITY, Jan. 1981, p. 413-418
0019-9567/81/010413-06$02.00

Vol. 31, No. 1

Evans Blue Dye Adjuvant Enhances Delayed Hypersensitivity While Blocking Immunity to *Mycobacterium tuberculosis* in Mice

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Evans blue dye functions as an adjuvant with protein antigens in saline to induce cell-mediated immunological responses in mice. But when used to help induce cell-mediated tuberculoimmunity, it decreased mouse resistance to tuberculosis instead of helping induce immunity. This paradox was investigated. As could be expected from previous work with other antigens, the dye did promote induction of delayed hypersensitivity in mice to tuberculoprotein when injected in saline with killed tubercle bacilli. Peritoneal macrophages from mice injected with the dye responded normally to migration inhibition factor. Morphologically, these cells were moderately "activated" compared with similar cells taken from untreated mice. However, such cells incubated with tuberculosis growth inhibition lymphokine in an in vitro test for tuberculoimmunity did not express tuberculoimmunity, whereas macrophages from untreated mice did. Therefore, Evans blue dye did promote induction of cell-mediated immunological responses and tuberculoimmunity in lymphocytes, but under the conditions used in these experiments, it also blocked expression of tuberculoimmunity by macrophages.

Evans blue dye (EB) is one of a few immunological adjuvants that, like Freund adjuvant, can help protein antigens induce cell-mediated (delayed) hypersensitivity (1). EB is a defined chemical which works in saline (i.e., without oil) and has been used harmlessly in man (as the drug T-1824 to measure plasma volume [18]).

The mouse model of tuberculosis was employed to study the effectiveness of EB as an adjuvant for an infectious agent. Intracellular infections with organisms such as *Mycobacterium tuberculosis* require lymphocyte and macrophage cooperation for controlling these organisms (22). Lymphokine(s) produced by lymphocytes in response to the organism stimulate macrophages to control the organisms they have phagocytized (6, 11).

In the experiments reported here, we show that when EB is used with an immunizing antigen to help induce cell-mediated tuberculoimmunity, it does the opposite and decreases mouse resistance to tuberculosis. Yet the mice that are not protected by the EB vaccine nevertheless demonstrate delayed hypersensitivity in vivo and in vitro. We go on to show that the reason for this unexpected result probably is that although EB may promote induction of tuberculoimmunity, presumably in lymphocytes, it blocks its expression in macrophages.

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MATERIALS AND METHODS

Animals. Female CF-1 mice, 8 to 12 weeks of age, were used in these experiments.

Reagents. EB {direct blue 53; 6,6'-[(3,3'-dimethyl[1,1'-biphenyl]-4,4'-diyl)bis(azo)]bis[4-amino-5-hydroxy-1,3-naphthalenedisulfonic acid] tetrasodium salt} was purchased from Matheson Coleman and Bell Manufacturing Chemists, Norwood, Ohio. Tissue culture media and reagents were purchased from GIBCO, Grand Island, N.Y., and Middlebrook 7H9 and 7H10 mycobacterial culture media were from Difco Laboratories, Detroit, Mich. Tissue culture media used were RPMI 1640 and Neuman-Tytell. Hanks balanced salt solution (HBSS) was used unmodified or with its $NaHCO_3$ replaced by 5 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Sigma Chemical Co., St. Louis, Mo.) (HEPES-HBSS). Kirchner mycobacterial defined surface culture medium was mixed in our laboratory by a previously described (7) formula. *n*-Hexadecane and glycerol monooleate (Eastman Kodak Co., Rochester, N.Y.) were used to make water-in-oil emulsion (w/o; 9). PBS refers to physiological saline buffered with sodium phosphate to pH 7.4.

Virulent *M. tuberculosis* Erdman and attenuated *Mycobacterium bovis* BCG were from stock cultures maintained in our laboratory. They were reactivated for use in mouse or tissue culture infection (i.e., the virulence of each was enhanced) by subculture from semisolid 7H10 medium onto Kirchner liquid medium (22). Thin-veil pellicular growth from the Kirchner medium then was used either directly for infecting mice or grown for 7 days in 7H9 medium for infecting macrophages in vitro. For immunization, Erdman bacilli harvested from Kirchner medium and killed in

acetone (8) were used. Tuberculoprotein used for footpad tests of tuberculin hypersensitivity and for migration inhibition factor (MIF) production was precipitated with ammonium sulfate from Erdman tubercle bacillus Kirchner culture medium filtrate (8). Trypsin-extracted tubercle bacillus immunizing antigen (TE) was prepared from acetone-washed BCG (8).

Induction and measurement of tuberculoimmunity in vivo. We used a survival method like that developed by Youmans and co-workers (22) for measuring tuberculoimmunity. Groups of 10 mice each were used. Mice were immunized subcutaneously on days 0 and 21 with acetone-killed bacilli or TE and then challenge infected on day 35. Those immunized with killed bacilli received 0.25 mg in 0.1 ml of either w/o emulsion or PBS containing 0.5 mg of EB. TE recipients were injected similarly but with 0.1 mg of TE in w/o or PBS-EB. One experiment included a group injected with PBS-EB without any antigen. Unimmunized mice were used as controls in all infection experiments.

Tuberculoimmunity in these variously treated mice was measured by challenging each mouse intravenously with a fine suspension of 0.25 mg of Erdman bacilli in 0.2 ml of PBS and then recording the time of the animal's death from tuberculosis (confirmed by autopsy) during the next 4 weeks.

Tuberculoimmunity in vitro. The principle for detecting tuberculoimmunity is to stimulate immune spleen lymphocytes to make immune lymphokine (growth inhibition factor [GIF], after Barksdale and Kim [2]), which appears to be different from MIF (22). Then, syngeneic tubercle bacillus-infected macrophages are cultured with GIF, which stimulates them either to arrest bacillary replication or kill the bacilli.

Spleen cells were prepared from mice 5 weeks after immunization (as described above) by pressing the spleen through a 60-mesh wire screen into ice cold HBSS. Erythrocytes were lysed by suspending the cells in 0.83% NH_4Cl-tris(hydroxymethyl)aminomethane preadjusted to pH 7.2. The nucleated cells remaining were washed twice on HBSS and suspended at 10^7/ml in 3 ml of Newman-Tytell medium without antibiotics but supplemented with 10% heated fetal calf serum and 100 μg of TE per ml. Conical plastic centrifuge tubes (no. 2095; Falcon Plastics, Oxnard, Calif.) containing the spleen cell-antigen cultures were incubated for 72 h at 37°C in air and 7% CO_2. After incubation, cultures were centrifuged at 300 × *g* and 4°C for 10 min, and supernatant fluid was drawn off and filtered through a 0.22-μm Millex-GS membrane filter (Millipore Corp., Bedford, Mass.) and stored at −70°C until tested. Since CF-1 mice are allogeneic, spleen cells from different individuals were incubated separately from each other.

To detect GIF in the lymphocyte supernatant fluids, their effects on the intracellular numbers of BCG in cultures of mouse peritoneal macrophages were measured by a method adapted from that of Cahall and Youmans (6; cf. 11). Briefly, 50-μl droplets of unstimulated mouse peritoneal cells (10^7 cells per ml) were plated on Falcon 3001 petri dishes and incubated for 30 min. Nonadherent cells were washed off, whereas the remaining adherent cells were incubated overnight in Neuman-Tytell medium containing 10% heated horse serum and 100 IU of penicillin per ml. The adherent cells (mostly macrophages, as judged by morphology) were infected by replacing the overnight medium with 1 ml of fresh medium containing 1.5 × 10^7 BCG cells per ml. After 30 min of incubation, the infected macrophages were washed vigorously with PBS warmed to 37°C. Several plates of infected macrophages were fixed for staining as zero-time controls, and 400 macrophages were evaluated to find the percent infected. To each of the remaining plates was added 1 ml of culture medium mixed 1:1 with spleen cell supernatant. Plates were also fixed and stained after 3 or 7 days of incubation. They were fixed in warm 0.25% glutaraldehyde in HEPES-HBSS and then stained by the Kinyoun modification of the Ziehl-Neelsen stain (17).

The method of Cahall and Youmans (6) was used to determine inhibition of mycobacterial growth within macrophages. The following formula was used as an indicator of mycobacterial growth.

$$\text{percent protection} = \left(\frac{\text{“under 10,” test}}{\text{“under 10,” control}} - 1.0\right) \times 100$$

The percentage of macrophages with fewer than 10 bacilli is a reflection of the activity of the lymphokine and the ability of macrophages to respond to GIF by inhibiting intracellular growth.

Measurement of tuberculin hypersensitivity in vivo. Tuberculin hypersensitivity was measured by a standard footpad swelling assay (13). A volume of 0.02 ml of a 0.1% (wt/vol) solution of tuberculoprotein was injected into the right hind footpad, whereas an injection of 0.02 ml of saline injected into the contralateral footpad served as the control. Dial-gauge calipers were used to measure the change in the thickness of tuberculoprotein-injected footpads compared with saline-injected footpads at 24 h as a measure of a specific hypersensitive reaction.

In vitro assay for tuberculin hypersensitivity. The tuberculin hypersensitivity assay was done by detecting MIF with a hanging drop procedure as previously described (10). The spleen cell culture fluids described above for GIF production and detection were tested separately for MIF. Briefly, peritoneal macrophages were washed from normal mice, washed twice, and suspended to 10^7 cells per ml in RPMI 1640 containing 10% fetal calf serum. Four 10-μl droplets were plated on each of several 35-mm petri dishes (Falcon; no. 1008) and incubated for 15 min. Afterwards, 50 μl of medium (RPMI 1640, 10% fetal calf serum, 10 μg of gentamicin per ml) with MIF or with control culture fluid from unstimulated spleen cells at 50% final concentration was gently overlaid on each droplet of cells. After another 15 min of incubation, each plate was inverted and again incubated for 15 min. At this time, the right-angle dimensions of the spots of adherent macrophages (most lymphocytes drop to the bottom of each hanging droplet in these upside-down plates) were measured at 40× with an ocular linear micrometer (no. 1046A; American Optical Corp., Buffalo, N.Y.). Each spot was measured in the same dimensions again after 24 h of incubation. Spot areas were computed by multiplying the measured

diameters and dividing the area after incubation by that obtained before incubation to get a migration ratio for indicating macrophage migration or inhibition of migration.

Adherent cell morphology was also examined in these plates after the 24-h diameters had been measured. The cells in each petri dish were fixed with warm 0.25% glutaraldehyde in HEPES-HBSS, washed, stained, and then counted. For these counts, each cell was classified as being dense, extended, intermediate (between extended and dense), or "activated" (i.e., with a fried-egg appearance; see reference 10 for an illustration of these cell types and their use in constructing a profile of adherent cell morphology).

RESULTS

Reduction of tuberculoimmunity by EB. Mice immunized with trypsin extract antigen mixed with EB in PBS were more susceptible to experimental tuberulosis than unimmunized mice, whereas mice immunized with this vaccine or killed tubercle bacilli in w/o emulsions developed immunity (Fig. 1). Mice injected with EB by the protocol of immunization (500 μg in PBS injected subcutaneously on days 0 and 21) but without any antigen showed the same increased susceptibility to tuberculosis in a separate experiment (Fig. 2). Taken together, these results suggested that EB was blocking expression, not development, of tuberculoimmunity because it acted in mice whether they received immunizing antigen or not. If so, it should not block development of other kinds of cell-mediated immunological responses to tubercule bacilli, for example, tuberculin hypersensitivity.

Induction of tuberculin hypersensitivity. Figure 3 shows that EB in PBS enhances induction by killed tubercle bacilli of tuberculin hypersensitivity in mice as compared with the use of the bacilli alone in PBS. These data, from two separate experiments, show that sensitization by the bacilli mixed with EB was as good as when they were injected in w/o emulsion.

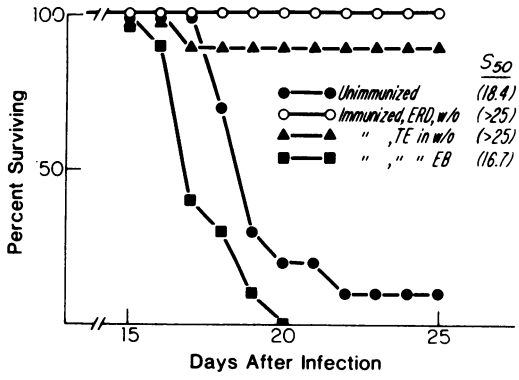

FIG. 1. *Survival curves and 50% survival values (S_{50}) in days after intravenous infection with 0.25 mg of Erdman tubercle bacilli for mice treated in various ways. These groups of 10 mice each were unimmunized (controls), immunized with acetone-killed Erdman bacilli in w/o, immunized with TE in w/o, or immunized with TE in an aqueous solution of EB as shown.*

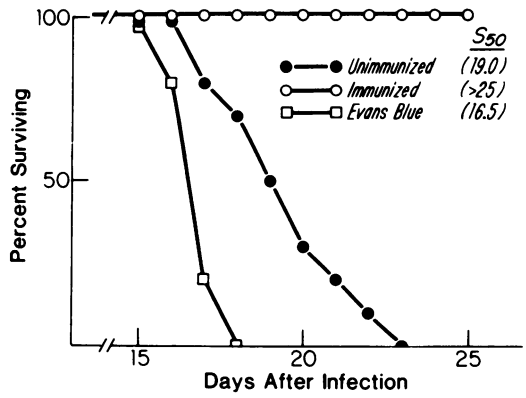

FIG. 2. *Survival curves and 50% survival values (S_{50}) in days in an experiment like that shown in Fig. 1 but with three groups of 10 mice; unimmunized, immunized with acetone-killed Erdman bacilli in w/o, and injected with EB solution as in the immunization of the fourth group in Fig. 1 but without antigen.*

Delayed hypersensitivity in vitro. The data in Table 1 concern two questions: do spleen lymphocytes from mice immunized by using EB as adjuvant make MIF when incubated with TE antigen, and can the peritoneal macrophages of mice that have been injected with EB migrate normally and be inhibited in their migration by, i.e., respond to, MIF normally? Results from the four separate experiments shown in Table 1 answer both questions affirmatively. Lymphocytes from mice immunized with TE in EB adjuvant do respond to TE in vitro by producing MIF. Furthermore, this lymphokine was effective on normal peritoneal macrophages as well as macrophages from mice treated with EB.

Morphology of peritoneal macrophages taken from mice injected with EB. Although peritoneal macrophages from EB-injected mice responded normally to MIF (Table 1), they were morphologically abnormal. This is shown in Fig. 4. Among adherent cells from EB-injected mice, well-spread (extended) cells predominated, whereas among adherent cells from normal mice, unspread (dense) cells predominated. This was true whether the cells were incubated for 30 min of overnight and whether they were taken from mice injected with EB at 3, 6, or 8 days before.

Tuberculoimmunity in vitro. The data above, plus a reasoning that induction and expression are different in tuberculoimmunity

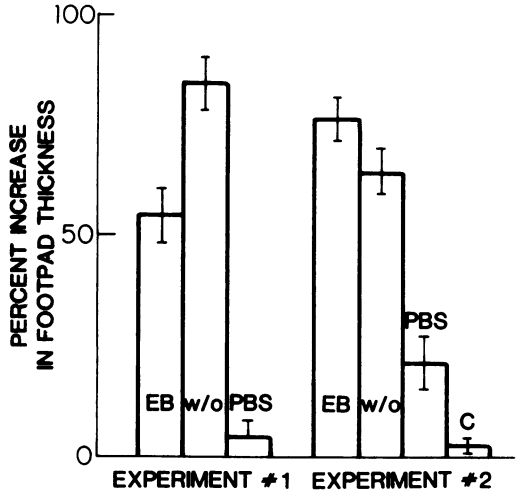

FIG. 3. *Footpad swelling tests for tuberculin hypersensitivity in mice immunized with acetone-killed bacilli in w/o, aqueous solution of EB, PBS, or no immunization (control). Footpads were measured 24 h after being injected with 0.02 ml of a 1% solution of tuberculoprotein. Data are expressed as the percent increase in thickness in the tested footpad over that of saline-injected footpad.*

TABLE 1. *Delayed hypersensitivity in vitro and capacity of peritoneal macrophages to respond to MIF*

Treatment of macrophage donors	Migration ratio[a]	
	Control culture	Antigen-exposed culture
EB subcutaneously, 3 days	1.17 (0.02)	1.00 (0.06)
Saline subcutaneously, 3 days	1.16 (0.01)	1.00 (0.01)
EB intraperitoneally, 6 days	1.17 (0.03)	0.96 (0.04)
Saline intraperitoneally, 6 days	1.21 (0.02)	1.00 (0.03)
EB subcutaneously, 6 days	1.24 (0.08)	0.96 (0.07)
Saline subcutaneously, 6 days	1.23 (0.06)	1.01 (0.04)
EB intraperitoneally, 8 days	1.13 (0.06)	0.93 (0.03)
Saline intraperitoneally, 8 days	1.11 (0.06)	0.93 (0.06)

[a] The values shown were calculated as described in the text and are the means of two experiments run in quadruplicate, with the standard error of the mean shown within parentheses. Control culture fluid (from lymphocytes from immune mice incubated without antigen) was compared with culture fluid from immune lymphocytes incubated with TE antigen. Donors of the immune lymphocytes had been immunized 5 weeks previously with acetone-killed bacilli in PBS and EB. TE itself does not inhibit macrophage migration (data not shown). Responding macrophages were obtained from mice previously injected with 0.1 ml of PBS or EB in PBS (500 μg in 0.1 ml) as indicated.

and between tuberculin hypersensitivity and tuberculoimmunity, suggested that measuring tuberculoimmunity of EB-injected mice in vitro could help explain why they seemed to lack tuberculoimmunity in vivo. Two kinds of experiments were done, one to test macrophages for response to GIF and the other to test lymphocytes for the ability to make it. The results summarized in Table 2 identify a defect in mice injected with EB that could account for their inability to express tuberculoimmunity: their macrophages not only did not respond defensively against infection with BCG, but actually became more susceptible than macrophages from unimmunized mice. According to results shown in Table 3, there was no defect in the spleen lymphocytes of mice immunized with bacilli mixed with EB, for they were able to make GIF as well as cells taken from mice immunized with bacilli injected in w/o.

DISCUSSION

Since EB is a good adjuvant for inducing tuberculin-type delayed hypersensitivity to various protein antigens in mice (1) and since it is a defined, water-soluble chemical, testing its usefulness for inducing cell-mediated anti-microbial immunities in mice seemed worthwhile. But when it was tried with tuberculoimmunity, it made mice more susceptible to tuberculosis, instead of helping to immunize them (Fig. 1 and 2). From studying this paradox, as we report

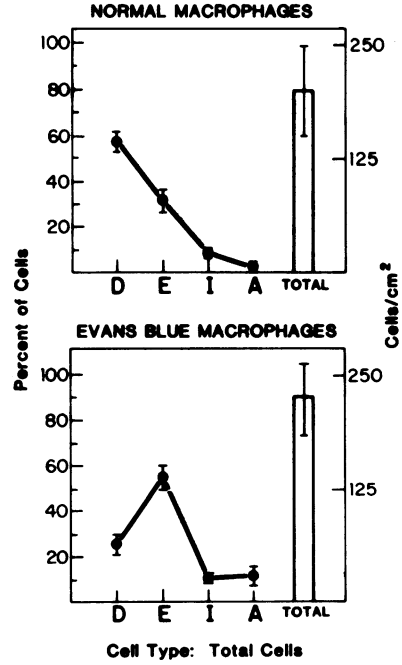

FIG. 4. *Cell profile of macrophages from normal mice (buffer injected) or mice injected 3 days before collection with 0.5 mg of EB dye subcutaneously. Cells were fixed, stained, and examined after 24 h of incubation in RPMI 1640 + 10% FCS. Bars represent standard error of the mean (n = 8). D, dense; E, extended; I, intermediate; A, activated. See reference 10 for an illustration of these cell types.*

TABLE 2. *Ability of macrophages from mice injected with EB to respond to GIF by expressing tuberculoimmunity in vitro*[a]

MØ	GIF	% MØ infected at day 0	Day 3 MØ with:		% Protection	Day 7 MØ with:		% Protection
			≤10 AFB	>10 AFB		≤10 AFB	>10 AFB	
EB	−	15	256	148		244	160	
EB	+	11	260	140	2	188	216	−23
Control	−	14	208	192		272	128	
Control	+	16	328	72	58	364	36	34

[a] Macrophages (MØ) taken from mice injected subcutaneously 3 days earlier with 0.5 mg of EB in PBS or from untreated mice (control) were infected with BCG on day 0 and then incubated in medium with or without GIF. Plates fixed and stained on days 0, 3, and 7 of the infection were counted to determine the percent infected on day 0 and the proportions of MØ infected with ≤10 or >10 acid-fast bacilli (AFB) per MØ. Fifty successive macrophages in each spot, two spots on one plate and two on another, were counted to make a total of four-hundred in four separate experiments. (See text for formula used to determine percent protection.) BCG incubated in GIF-containing medium without macrophages demonstrated enhancement of growth (data not shown).

TABLE 3. *Ability of spleen lymphocytes from mice immunized with killed Erdman bacilli, using EB adjuvant, as compared with w/o emulsion to make GIF when incubated with TE immunizing antigen*[a]

Source of spleen cells	Percent MØ infected at day 0	Day 3 MØ with:		% Protection	Day 7 MØ with:		% Protection
		≤10 AFB	>10 AFB		≤10 AFB	>10 AFB	
Erdman + w/o	34	172	28	26	172	14	13
Erdman + EB	35	176	24	29	180	22	18
Nonimmunized	33	136	66		152	50	
None (medium only)	37	144	58	5	150	25	−1

[a] Spleen cells from mice immunized with acetone-killed Erdman bacilli in w/o or in PBS-EB 5 weeks earlier were incubated with TE for 72 h, and their supernatant culture fluids were tested for GIF activity by comparison with similar culture fluid from spleen cells of nonimmunized mice also incubated with TE. See Table 2, footnote *a*, for an explanation of the numbers and abbreviations. Data represent two separate experiments run in duplicate for a total of 200 cells counted per group.

here, we believe that this occurred because of the dye's interference with expression of tuberculoimmunity by macrophages.

Tuberculin hypersensitivity and tuberculoimmunity are similar cell-mediated immunological responses with different lymphokine mediators (22). Induction of tuberculin hypersensitivity was enhanced by EB injected in saline with killed tubercle bacilli, as tested both by footpad challenge in mice (Table 1) and by MIF production and detection in vitro (Table 2). Thus, considering tuberculin hypersensitivity as an example, EB was enhancing, not inhibiting, induction of cell-mediated responses to tubercle bacillus antigens and the stimulation of lymphokine-producing lymphocytes. Therefore, it was not interfering with the lymphocyte arm of the cell-mediated immunological response.

According to these experiments with tuberculin hypersensitivity, there was also no defect in the peritoneal macrophages of EB-injected mice, for they responded to MIF. In fact, they looked somewhat activated (Fig. 4). But the function of a macrophage detecting MIF is passive (inhibited migration), whereas it has an active (antimicrobial) function in tuberculoimmunity. When mouse tuberculoimmunity itself was studied in vitro, where the functions of lymphocytes and macrophages could be separated from each other (Tables 2 and 3), the lymphocytes from mice injected with EB and immunizing antigen acted immune and produced immune lymphokine (i.e., GIF). But macrophages from the EB-injected mice were defective; they could not respond to GIF to inhibit or kill tubercle bacilli they had ingested. Thus, EB does help induce tuberculoimmunity in mice, but this immunity cannot be expressed because the dye also blocks some essential antimicrobial function of the macrophages in these mice.

EB, and particularly its isomer, trypan blue dye, are well known to accumulate selectively in the lysosomes of monocytic phagocytes (1, 4, 13, 20), where they inhibit lysosomal enzymes (4, 12) and, thus, also certain macrophage functions. For trypan blue, these include expression of cell-mediated tumor immunity, allograft rejection, and cytotoxicity (14–16); for both, they include tissue reconstruction, which is a reason that they are teratogenic (3). The principal difference between these two dyes biologically, which makes EB (and chemically related suramin) an adju-

vant but trypan blue not an adjuvant (1), is the much longer persistence in vivo of EB (1, 3), partly because it binds much more strongly to serum α_1-lipoprotein (18, 19). Because of this, EB, unlike trypan blue, can seem to have the opposite effects of either enhancing or inhibiting cell-mediated immunological responses, depending on experimental timing and criteria.

Immunological adjuvants are well known to have potentially opposite effects (21), and the data here show why for one of them. The impairment of a macrophage function (digestion) that imparts adjuvanticity (1) also can block expression of the immune response being induced, due to long-term persistence in the body of the adjuvant. This shows why criteria by which adjuvanticity is measured are so important, for in these experiments two expressions of the same immunological response (GIF production and GIF expression) gave opposite results (5). Even with one criterion, our results might have been different if we had waited for several months to challenge mice immunized with EB, when they would have had enough unblocked macrophages to have expressed tuberculoimmunity. Our experiments present an additional puzzle; if the macrophages of EB-injected mice were unable to express cellular immunity, and if cellular immunity is an everyday barrier against infectious disease, how did the mice protect themselves from their environment? But since they did, remaining healthy until they were infected with tubercle bacilli, EB may have been blocking only a few macrophage defense mechanisms. If so, EB and related chemicals, like trypan blue (16), should be useful for refined probing of the expression of certain antimicrobial immunities like tuberculoimmunity.

ACKNOWLEDGMENTS

We thank Albert Zlotnik for his help in setting up the GIF experiments.

This research was supported by the Harry Winston Foundation.

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