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Glucan-Enhanced Immunogenicity of Killed Erythrocytic Stages of *Plasmodium berghei*

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Intravenous injections of glucan simultaneously with Formalin-killed erythrocytic stages of *Plasmodium berghei* elicited a greater degree of resistance in mice against subsequent infection with viable parasites than injections of killed erythrocytic stages alone. In two experiments with *P. berghei* strain NK 65, 100% of mice immunized with the glucan-dead parasite preparation survived challenge, whereas only 28.6% of mice receiving dead parasites alone survived. In the third experiment, using *P. berghei* strain NYU-2, the same proportion of mice survived after immunization with glucan and dead parasites as with dead parasites alone (i.e., 10 of 11 in each group), but mice immunized with the glucan-dead parasite preparation experienced parasitemias of significantly less intensity and shorter duration than mice which received only dead parasites before infection. Inoculation of glucan alone or with normal erythrocytes conferred no protection against challenge.

Glucan is a polysaccharide constituent of the inner cell wall of bakers' yeast, *Saccharomyces cerevisiae*, consisting of a chain of glucopyranose units united by a β1,3-glucosidic linkage. Stimulation with glucan elicits nonspecific resistance in experimental animals against several infectious agents, including *Candida albicans* (15), *Sporotrichum schenkii* (M. Stevens, P. Stevens, J. A. Cook, H. Ichinose, and N. R. Di Luzio, RES J. Reticuloendothel. Soc. **20**:66A, 1976), *Mycobacterium leprae* (3), *Staphylococcus aureus* (7), murine viral hepatitis (18), and *Leishmania donovani* (2).

Recent studies have shown that glucan exerts an adjuvant effect in experimental immunization trials in visceral leishmaniasis (T. W. Holbrook, J. A. Cook, and B. W. Parker, Am. J. Trop. Med. Hyg., in press) and Venezuelan equine encephalitis (14). In this report, we describe a strong adjuvant effect of glucan injected simultaneously with Formalin-killed erythrocytic stages of the rodent malaria parasite *Plasmodium berghei*.

MATERIALS AND METHODS

Parasite and experimental animals. *P. berghei* strain NK 65 was used in the initial two experiments in the study. The strain was obtained from Richard Roth, Rutgers University, and has been maintained here by serial passage in mice for several years. For a third experiment, *P. berghei* strain NYU-2 was recently acquired as a frozen stabilate from the American Type Culture Collection (no. 30090). The parasite strain is presently maintained by serial intraperitoneal passage of infected blood in mice. Female ICR mice were obtained from the Department of Laboratory Animal Medicine at this institution. Animals were 8 weeks old at the start of experiments and were housed in air-conditioned quarters; they were allowed food and water ad libitum.

Immunization method. Parasitized erythrocytes (RBC) used for immunization were obtained by retroorbital bleeding of mice exhibiting at least 50% parasitemia. RBC were washed three times in Earle balanced salt solution and killed by suspension in 0.1% Formalin for 30 min at room temperature and kept at 4°C overnight. Cells were washed three times and resuspended in Earle balanced salt solution at a concentration of 10^8 Formalin-killed parasitized RBC (FKP) per 0.2 ml. Normal RBC were obtained from uninfected mice and treated in the same manner.

Glucan was prepared from bakers' yeast (*S. cerevisiae*) by a modification of the method of Hassid et al. (6). Briefly, yeast cells were digested in 3% NaOH in distilled, deionized water (100°C, 4 h). The procedure was repeated twice, and the residue was acidified with 3% HCl (100°C, 4 h). That procedure was repeated twice, and the suspension was centrifuged. The centrifugate was washed in distilled, deionized water and extracted with ethanol. The ethanol was removed by washing at least five times in distilled water. For use in these studies, glucan was suspended in sterile, pyrogen-free 5% dextrose at a concentration of 0.45 mg/0.2 ml. For simultaneous injections, 0.2-ml suspensions of glucan (0.45 mg) and Formalin-treated parasitized or normal RBC (10^8) were mixed just before injection. For injections of cells or glucan alone, the volume of each was adjusted so that all animals received 0.4 ml per injection. All immunization injections were via the intravenous (i.v.) route.

Hematological and parasitological parameters. Thin blood smears and hematocrits were prepared at appropriate times from tail snips of each

mouse during the immunization procedure and after challenge with viable parasites. Blood smears were fixed with methanol and stained with Giemsa. Degree of parasitemia and percentage of immature (i.e., basophilic) RBC were determined by counting at least 250 RBC.

RESULTS

Experiments with *P. berghei* strain NK 65. In two separate experiments, mice received treatments presented in Table 1. In the first experiment, we tested the response to infection of animals pretreated by i.v. injections of FKP in combination with glucan. Another group of mice received injections of FKP alone, and an untreated control group was included. In the second experiment, the same immunization schedule was followed by additional groups of mice received normal RBC (NRBC) with or without glucan. The effects of glucan alone and of 5% dextrose were tested in the second experiment.

An important consequence of i.v. injection of glucan alone or simultaneously with either FKP or NRBC was a decrease in packed RBC volume (Fig. 1) and compensatory reticulocytosis. Lowest hematocrit values were seen 6 days after the final injection of glucan, and immature RBC represented up to 18% of all RBC in some mice in those three groups. Mice receiving FKP, NRBC, or dextrose alone exhibited no significant change in hematocrit value nor in percentage of immature RBC during the immunization schedule. The increase in the proportion of immature RBC in glucan recipients was an important consideration in testing the response of mice to challenge with viable parasites since the parasite preferentially invades reticulocytes. Recovery of normal packed cell volume and reticulocyte percentage required about 3 weeks after the final immunizing injection in glucan recipients.

TABLE 1. *Schedule for i.v. immunization of mice against P. berghei erythrocytic stages*

Group	Injection on given day[a]				
	0	2	4	6	8
FKP + Glu	FKP, Glu	Glu	FKP, Glu	Glu	FKP, Glu
FKP	FKP	NT	FKP	NT	FKP
NRBC + Glu	NRBC, Glu	Glu	NRBC, Glu	Glu	NRBC, Glu
NRBC	NRBC	NT	NRBC	NT	NRBC
Glu	Glu	Glu	Glu	Glu	Glu
D	D	D	D	D	D
NT	NT	NT	NT	NT	NT

[a] FKP, 10^8 Formalin-killed parasitized erythrocytes; Glu, 0.45 mg of glucan; NRBC, 10^8 Formalin-treated normal erythrocytes; D, 5% dextrose; NT, no treatment.

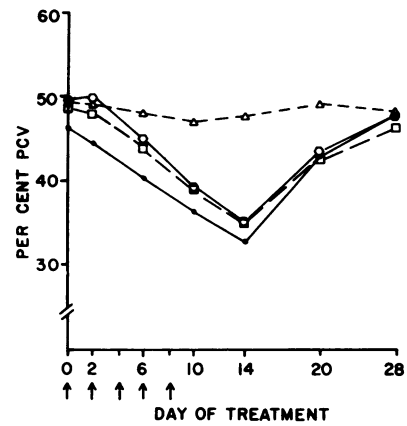

FIG. 1. *Change in packed RBC volume in groups of mice receiving glucan alone (◇) or with dead parasitized (●) or normal erythrocytes (□). Hematocrit values of glucan recipients were significantly less ($P < 0.001$) than those of untreated mice (△) on days 10, 14, and 20.*

Challenge was by i.v. injection of 5.0×10^5 parasitized RBC on day 34 after the final immunization in experiment 1 and on day 29 in experiment 2.

In each experiment, all mice which had been pretreated with FKP and glucan (group FKP+Glu) survived infection. Only 4 of 14 mice which had been injected with FKP alone in the two experiments survived, and all animals in other groups died (Table 2). During the first few days after challenge, parasitemias in FKP+Glu animals and mice in other groups were similar (Fig. 2). A mean peak parasitemia of 17.6% was recorded on day 10 postinfection in FKP+Glu mice in experiment 2. Parasites were no longer seen in blood smears from 10 of 13 FKP+Glu mice after 14 days postinfection in the two experiments, and parasitemias in the remaining three mice in that group subsequently decreased to microscopically undetectable levels. In contrast, parasitemias continued to increase in unprotected animals. Several mice pretreated with FKP alone exhibited a transient decrease in parasitemia but later experienced an increase in the proportion of parasitized RBC and subsequently died. No evidence of recrudescence was seen after the initial parasitemia decrease in FKP+Glu mice.

Thirty-six days after the initial challenge in experiment 1, 0.1 ml of whole blood was subinoculated from each recovered animal (seven in group FKP+Glu and one in group FKP) into a previously uninfected mouse to determine if subpatent parasitemia could be demonstrated in recovered animals. Five of seven recipients of blood from FKP+Glu mice remained uninfected

TABLE 2. *Result of challenge of mice immunized against P. berghei strain NK 65 RBC stages*[a]

Expt	Group	No. of mice	% Survivors	Mean time to death (days)[b]
1	FKP + Glu	7	100	
	FKP	7	14.3	32.2 (8.2)
	NT	7	0	17.7 (8.2)
2	FKP + Glu	6	100	
	FKP	7	42.9	12.8 (7.8)
	NRBC + Glu	7	0	16.7 (3.6)
	NRBC	7	0	15.1 (5.2)
	Glu	7	0	15.0 (3.3)
	D	7	0	17.0 (4.9)
	NT	6	0	17.0 (3.8)

[a] For immunization schedule and group treatments, see Table 1. Mice were challenged on day 34 after last immunization injection in experiment 1 and on day 29 in experiment 2. Each animal received 5×10^5 erythrocytic stages i.v.

[b] Standard deviations are given in parentheses.

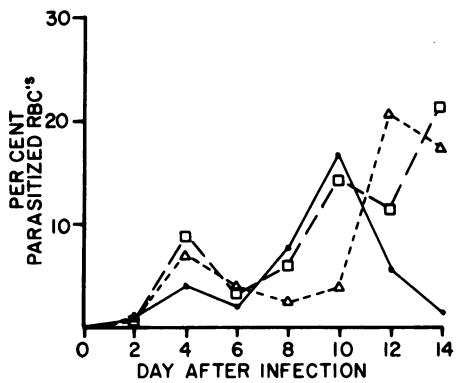

FIG. 2. *Early course of P. berghei (strain NK 65) infection in mice pretreated with FKP+Glu (●), or FKP alone (□) or untreated before challenge (△). For group treatment, see Table 1. Mice received 5×10^5 parasitized RBC on day 0. After day 14, parasitemias in FKP+Glu mice decreased, whereas those of untreated mice and most FKP mice increased. Parasitemias for mice in other control groups (see Table 1) were similar to those of untreated mice.*

(blood films examined for 28 days). Blood from two mice in group FKP+Glu and the single FKP survivor produced infection in recipients. Four days after removal of blood for subinoculation (i.e., day 40 after initial challenge), the recovered FKP+Glu and FKP mice received a second i.v. challenge (10^7 parasitized RBC). Four of seven FKP+Glu mice exhibited no parasite in daily blood films after the second challenge. The maximum parasitemia seen in that group was 0.4% in one mouse on day 6 postchallenge. The single FKP animal experienced a peak parasitemia of 7.8% but recovered. Two age-matched, previously untreated mice infected at the same time died on days 20 and 21, respectively.

Blood subinoculation and challenge of recovered mice in experiment 2 produced a result similar to that of the first experiment. One of six mice became infected after receiving 0.1 ml of whole blood from FKP+Glu mice 42 days after those animals had received the initial challenge. Blood from all three mice which survived infection after being immunized with FKP alone produced infection in recipients. All FKP+Glu and FKP mice were resistant to a second challenge. Each animal received 10^8 parasitized RBC i.v. 44 days after the first challenge. One FKP+Glu animal exhibited a peak parasitemia of 1.2% on day 6 after the second challenge. Parasitemias of other mice in the two groups never exceeded 0.4%.

Experiment with *P. berghei* strain NYU-2. Thirty mice were allotted to three groups. Eleven mice received the combined glucan-dead parasite (strain NYU-2) regimen according to the schedule used in previous experiments (group FKP+Glu; Table 1). Eleven mice received killed parasites alone (group FKP), and eight mice were not treated before challenge (group NT). Parasites used for immunization were collected from mice one passage after establishing infection from a frozen stabilate. Five days after completion of the immunization procedure, hematocrit values of group FKP+Glu mice (mean, 41.1) were significantly lower ($P < 0.005$) than in groups FKP and NT (means, 50.6 and 49.5, respectively). Packed cell volumes of FKP+Glu animals increased thereafter, and by day 15 after immunization the mean hematocrit value of group FKP+Glu mice was similar to that of other groups. Challenge was by i.v. injection of 5.0×10^5 parasitized RBC (strain NYU-2) 21 days after the last immunization injection. The parasites used for challenge had been passaged four times in mice after initiation of infection with the frozen stabilate. Table 3 summarizes the results.

Parasitemias of mice in group FKP+Glu were similar to those of animals in groups FKP and NT until day 8 postchallenge. A single mouse in group FKP+Glu died on day 7 with 43.9% parasitized RBC, but the highest parasitemia recorded among the 10 survivors in the group was 13.5% in one animal. After day 8 postinfection, parasitemias of group FKP+Glu survivors decreased, and by day 14 no parasite was seen in slides from those mice. In contrast, parasitemias of group FKP and NT animals increased to a higher level and persisted for a longer period than in FKP+Glu mice. Only one mouse in group FKP died after challenge, but survivors in the group exhibited a significantly higher mean

TABLE 3. *Result of challenge of immunized mice with erythrocytic stages of P. berghei strain NYU-2*[a]

Group	No. of survivors/ no. challenged	Days to clearance to parasitemia[b]	Mean peak parasitemia[c]
FKP + Glu	10/11	8.6 (2.1)	5.1 (4.1)
FKP	10/11	25.9 (5.6)	52.1 (7.7)
NT	2/8	36.5 (7.8)	56.8 (14.1)

[a] Each mouse received 5.0×10^5 parasitized RBC i.v. 21 days after immunization (see Table 1 for immunization schedule).

[b] From day of infection to last day of microscopic patency; standard deviations are given in parentheses.

[c] Survivors only; standard deviations are given in parentheses.

peak parasitemia ($P < 0.005$) and patent infections of longer duration ($P < 0.005$) than group FKP+Glu mice. Parasitized RBC were seen in blood films from each survivor in group FKP for at least 20 days after challenge. Six of eight untreated mice (group NT) died after challenge, and the two surviving mice in that group exhibited patent infections for 31 and 38 days, respectively.

Thirty-six days after challenge, 0.1 ml of blood was subinoculated from each animal with no detectable parasites into a previously uninfected mouse. All recipients of blood from group FKP animals and one group NT survivor became infected. Two mice which received blood from group FKP+Glu mice became infected, but eight recipients of blood from mice in that group remained uninfected (last examined 28 days after inoculation).

Immediately after blood was harvested for subinoculation, each mouse in groups FKP+Glu, FKP, and NT received a second challenge. Each mouse was inoculated i.v. with 10^8 parasitized erythrocytes and exhibited strong resistance to the second challenge. The highest parasitemia recorded was 4.1% in a group FKP mouse. Other mice challenged at that time either remained subpatent or exhibited a maximum parasitemia of 0.4%.

DISCUSSION

Results of this study clearly demonstrate an adjuvant effect of glucan injected with dead erythrocytic stages of two strains of *P. berghei* in ICR mice. With strain NK 65 (experiments 1 and 2), only 4 of 14 mice immunized with FKP alone survived challenge, whereas all mice receiving both FKP and glucan survived (Table 2). Glucan injected alone or with NRBC provided no protection. The result of experiment 3 was less definitive since as many mice immunized with dead parasites of strain NYU-2 survived as animals which had received the combined dead parasite-glucan preparation (i.e., 10 of 11 mice in each group). The NYU-2 strain was less virulent for ICR mice in that experiment than previously reported (11), perhaps as a consequence of using parasites for immunization and challenge soon after establishing infection in mice from a frozen parasite stabilate. The adjuvant effect of glucan was evident, however, since FKG+Glu survivors experienced parasitemias of significantly lower intensity and shorter duration than mice which had received dead parasites alone (Table 3).

Results of this study are similar in some respects to recent reports demonstrating immunogenicity of Formalin-killed malaria parasites of rodents. Playfair et al. (12) and Murphy and Lefford (10, 11) demonstrated that mice could be protected against otherwise lethal infection by immunization with Formalin-treated erythrocytic forms of *P. yoelii* and *P. berghei*, respectively. In those studies, as in the present report, parasitemias of immunized and control mice were similar during the first few days after challenge. It is interesting to note that inclusion of the microbial immunopotentiator *Bordetella pertussis* with killed *P. yoelii* blood forms accelerated the rate of parasitemia clearance after challenge with that parasite (12) and that pretreatment with BCG enhanced resistance conferred by dead RBC stages of *P. berghei* (10).

Glucan has certain advantages in comparison with microbial agents used to enhance immune responses. Glucan is nonviable, chemically defined, and nonantigenic. In addition to inducing proliferation of macrophages in liver, lung, and spleen, glucan enhances both cell-mediated and humoral immune responsiveness (5, 9, 17, 18). Pertinent to its possible practical use, it is important to note that glucan has received evaluation in treatment of human malignant melanoma. Intralesional inoculation of glucan often produced prompt resolution without significant toxicity in most patients (8).

Glucan was recently shown to have an adjuvant effect when used in immunization trials against protozoan and viral agents. Mice which had been immunized with killed *L. donovani* promastigotes in combination with glucan were more resistant to challenge than animals which were injected with glucan alone, whereas dead promastigotes alone conferred no protection (Holbrook et al., in press). Reynolds et al. (14) found that a greater proportion of mice receiving glucan with Venezuelan equine encephalitis virus vaccine survived challenge than animals immunized with vaccine alone or combined with complete Freund adjuvant. Also, monkeys immunized with glucan and Venezuelan equine

encephalitis virus vaccine had higher antibody levels than animals which received only vaccine (14). Results described in those reports and the present study suggest that glucan should be tested for an adjuvant effect in other host-parasite systems.

Certain features of the results described in this report must be considered in relation to the potential practical application of glucan as an adjuvant for immunization. The response of rodents to adjuvant-enhanced immunization with nonviable malaria antigens appears to differ from that of primates. For example, complete Freund adjuvant injected with dead merozoites strongly enhanced the response of monkeys against challenge with *P. knowlesi* erythrocytic stages (1), but complete Freund adjuvant was not effective in immunization trials against rodent malaria (4, 13). Glucan has been shown to enhance responsiveness of monkeys immunized with a viral vaccine (14), but studies of other host-*Plasmodium* systems will be needed to determine whether glucan may have an adjuvant effect with dead malaria parasites in primates. The possible necessity for intravascular stimulation, consequent transient anemia during immunization, and the development of parasitemia early after challenge are potential limiting factors in applying the method in other systems. Further study is needed to determine whether a lower concentration of glucan with dead parasites will elicit protection equivalent to that seen in these experiments and whether use of alternative immunization routes will be effective.

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