Glucan as an Adjuvant for a Murine Babesia microti Immunization Trial

JORGE L. BENACH,1,2* GAIL S. HABICHT,2 THOMAS W. HOLBROOK,3 AND JAMES A. COOK4

State of New York Departments of Health1 and Pathology,2 State University of New York at Stony Brook, Stony Brook, New York 11794, and Departments of Laboratory Medicine3 and Physiology,4 Medical University of South Carolina, Charleston, South Carolina 29425

Received 3 August 1981/Accepted 30 October 1981

A vaccination protocol against murine Babesia microti infection, using glucan, a β-1,3-glucopyranose derivative of yeast cell walls, and glutaraldehyde-fixed infected erythrocytes was evaluated. BALB/c mice were immunized intravenously four times at 2-day intervals with 2 × 10⁸ fixed infected erythrocytes with and without glucan (0.45 mg). The mice were challenged 2 weeks after the last immunization with 10⁸ viable infected erythrocytes. Peak parasitemia was significantly reduced (8.9 ± 1.0%; P < 0.001) in glucan-immunized mice as compared with nonimmunized controls (41.2 ± 1.4%), glucan-treated controls (31.7 ± 2.5%; P < 0.05), or mice which received fixed infected erythrocytes without glucan (21.0 ± 1.2%; P < 0.01). Humoral and cellular immunity to B. microti was evaluated before challenge by measuring antibody titers and splenocyte blastogenic responses to B. microti antigens. The in vitro cellular response was inversely correlated with mean peak parasitemia (P < 0.05). These observations demonstrate that glucan is an effective adjuvant in enhancing immunity to murine babesiosis.

Babesiosis is best known as a severe disease of domestic cattle which causes sizable economic losses. Attempts to control the spread of babesiosis in cattle have been made for a long time with variable success (19). Most recently, available cultures of cattle Babesia (17) have made successful immunizations easier to achieve by using a culture-derived immunogen and saponin as an adjuvant (23).

In addition to Babesia species affecting domestic large animals, Babesia microti, a parasite of wild rodents, is now known to be the etiological agent of clinical babesiosis in humans in the United States (8). B. microti has been adapted to inbred laboratory mice, thereby providing a host-parasite model suitable for immunological and protection studies (22).

The need for efficient adjuvants for the preparation of vaccines to intraerythrocytic parasites is an important component in the development of successful protection. We present here the results of a vaccine trial with glutaraldehyde-fixed infected erythrocytes (RBC) as antigen and glucan as an adjuvant in a B. microti-BALB/c mouse model. Glucan is a β-1,3-glucopyranose derivative of yeast cell walls and a proven stimulant of the reticuloendothelial system. Glucan treatment has conferred nonspecific protection against a diverse group of viral (27), fungal (26), bacterial (16), and parasitic (6) infections. Glucan has been used successfully as an adjuvant in trials with Venezuelan equine encephalitis virus (20) and Plasmodium berghei (13) and also has been used in combination with glutaraldehyde-treated tumor cells for induction of immunity to murine leukemia (1).

MATERIALS AND METHODS

B. microti and mouse strains. The human-derived Peabody strain of B. microti was supplied by Mary Ruebush of the Bowman-Gray School of Medicine, Winston-Salem, N.C., and maintained in our laboratory by blood passage in BALB/c mice. For these trials, male BALB/c mice weighing 18 to 25 g each were used as donors of the antigen preparation and for the vaccine trials.

Preparation of inoculum. Glucan was prepared by a modification of the technique of Hassid and co-workers (12), resulting in a sterile suspension of glucan in 5% dextrose. B. microti-infected RBC to be used as antigen were collected from mice with parasitemias of approximately 50%; the plasma and as much of the buffy coat as possible were removed, and the RBC were washed three times in sterile phosphate-buffered saline (pH 7.2). After the last wash, the cells were fixed in 0.25% glutaraldehyde for 15 min at room temperature, followed by three additional washes in sterile phosphate-buffered saline supplemented with penicillin and streptomycin. The fixed RBC were stored at 4°C in sterile 5% dextrose supplemented with antibiotics until used. Just before inoculation, the cells were washed twice in sterile 5% dextrose, counted, and reconstituted at the desired concentrations. Preliminary investigations showed that corresponding numbers of fixed normal RBC alone or with glucan as well as 5% dextrose alone had no protective effect and are not reported here.
Immunization protocol. In two separate experiments, a total of 168 mice were divided into four groups of 21 mice each for each of the two experiments and immunized intravenously four times on alternate days. One group served as untreated, nonimmunized controls. A second group received 0.45 mg of glucan in 0.2 ml of sterile 5% dextrose. A third group received 2 × 10^6 glutaraldehyde-fixed infected RBC (IRBC) in 0.2 ml of 5% dextrose (IRBC group). The last group received 2 × 10^6 glutaraldehyde-fixed IRBC (G-IRBC) and 0.45 mg of glucan in 0.3 ml of 5% dextrose (G-IRBC group).

Determination of immunization effectiveness. At 2 weeks after the last inoculation, 16 mice from each group (8 from each experiment) were sacrificed to determine specific antibody production, cellular response, and hematological values after the vaccine regimen. The serum was used for determination of antibodies specific for B. microti antigens by indirect immunofluorescence (3), using an anti-mouse immunoglobulin G conjugate (Cappel Laboratories). Hematrits and leukocyte counts were made in a Coulter Counter (Model MCV-HCT; Coulter Electronics). Leukocyte differentials were made by conventional staining and microscopy.

Immediately after exsanguination, the spleen was removed aseptically and teased in RPMI 1640 medium supplemented with antibiotics to make a single-cell suspension. The splenocytes were washed three times, counted, resuspended in culture medium (RPMI 1640, 10 mM NaHCO_3, 20 mM HEPES [N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid], 2.5% fetal calf serum, penicillin, streptomycin), and dispensed into sterile, flat-bottomed microtiter plates (Linbro) at 7.5 × 10^3 splenocytes per well. Antigen-specific responses were measured by adding 10^6 washed IRBC (45 to 55% parasitemia) per well; an equal number of normal mouse RBC were used as the antigen control (25). Mitogens used to assess splenocyte function were phytohemagglutinin P (PHA; Sigma Chemical Co.), concanavalin A (ConA; GIBCO Laboratories), and Escherichia coli lipopolysaccharide (LPS) 0111-B4 (Westphal preparation; Difco). PHA was used at final well concentrations of 1:200, ConA was used at 5 μg/ml, and LPS was used at 20 μg/ml. The cultures were incubated at 37°C in a 5% CO_2 atmosphere and harvested at 96 h after having been pulsed 18 to 24 h earlier with 1 μCi of [3H]thymidine. Cultures were harvested by means of a multiple-sample automated cell harvester on glass filter paper disks. The filter paper disks were treated with methanol and Hyamine hydroxide (National Diagnostics) and then with 3 ml of scintillation fluid before being counted in a liquid scintillation counter (Mark III Beta; Tracor Analytic). Incorporation of [3H]thymidine was expressed as mean disintegrations per minute (DPM) ± standard error of the mean of quintuplicate cultures. Stimulation indices were calculated as mean DPM divided by mean DPM of unstimulated control cultures containing serum alone. All statistical analyses were done by Student's t test.

Challenge. The remaining immunized mice and controls (26 per group) were challenged intraperitoneally with 10^6 parasitized RBC. Parasitemias (percentage of RBC infected) were determined daily until no parasites could be found in the Giemsa-stained peripheral blood smears. Initial experiments in which glucan alone (0.45 mg for 4 days) was used and the challenge dose was lowered to 5 × 10^7 and 1 × 10^6 parasitized RBC did not result in reduction of parasitemia and are not reported.

RESULTS

Immunization of mice with a combination of G-IRBC and the glucan adjuvant affords a significant degree of protection against challenge with a large number (10^8) of B. microti-parasitized cells (Fig. 1). Mean parasitemias in the mice of the G-IRBC group were significantly different from the glucan and control groups from days 5 to 8 (P < 0.001) and also significantly different from the IRBC group on days 5 to 7 (P < 0.05). The highest parasitemia recorded for any individual mouse in the G-IRBC group was 14% on day 5. IRBC or glucan alone provided only slight protection as compared with the nonimmunized controls: P < 0.01 (days 6 and 7) and P < 0.05 (days 6 through 10), respectively.

Predictors of protection against challenge. Regression analysis showed a significant (P < 0.05) inverse relationship between mean splenocyte response to the specific antigen (Table 1) and the highest parasitemia recorded after challenge for the mice of the same group (Fig. 1); that is, an increase in the [3H]thymidine uptake (DPM) in the sacrificed mice was associated with a decrease in the parasitemia in the remaining mice from the same group who were subsequently challenged. The same analysis showed a similar inverse relationship between the mean stimula-

![FIG. 1. Mean percent parasitemia (± standard deviation) after challenge of control and vaccinated mice with 10^8 parasitized RBC. Symbols: ○, control mouse; ▲, mice immunized with glucan; ○, IRBC group; △, IRBC plus glucan.](http://iai.asm.org/..)
tion indices (Table 1) and mean parasitemia. Thus, the greater antigen-specific stimulation seen in the mice of the G-IRBC and IRBC groups was found to have predictive value in assessing the existence of in vivo protection against challenge. Antibody titers (Table 2) in mice from the G-IRBC and IRBC groups were also significantly and inversely associated with a decrease in mean parasitemias of these mice as compared with those of controls or mice treated with glucan alone. However, there was no difference in antibody titers between mice from the G-IRBC and IRBC groups.

Responses to nonspecific lymphocyte mitogens. Although a decrease in splenocyte responsiveness to PHA was seen in the mice of all treated groups, only one significant deviation ($P < 0.02$) was seen in the mean response of the mice from the G-IRBC group to PHA (Table 1). The mean response of the splenocytes from the glucan-treated mice to ConA was significantly elevated ($P < 0.05$) in comparison with all of the other groups. A significant reduction in $[^{3}H]$thymidine uptake to ConA was noted in the mice from the IRBC group. Nonsignificant decreases in $[^{3}H]$thymidine uptake in the LPS response were noted in the splenocytes from the mice from the IRBC and G-IRBC groups.

Glucon treatment, antigen immunization, and glucan and antigen combined resulted in increases in the numbers of cells in the spleen, with a significant increase ($P < 0.05$) in the mice from the G-IRBC group. A decrease in hematocrit occurred in all of the treated mice (Table 2). Leukocyte counts were within normal limits. Differentials were also within normal limits; however, slight monocytosis, defined as 10 to 12% of the differential, was seen in 32% of the glucan-treated mice and in 37% of mice from the G-IRBC group. Reticulocytosis (5 to 12%) was seen in all treated mice.

**DISCUSSION**

Immunization of mice with a combination of glutaraldehyde-fixed, *Babesia* -infected RBC and glucan as an adjuvant affords a significant degree of protection against challenge with a large number of viable parasites ($10^8$ per mouse). A deliberately high challenge dose was used, since mortality of the experimental animal in this *B. microti* mouse model is not a criterion that can be used in the measurement of protection (22). This immunization trial also provided evidence that prechallenge antibody and cellular immunity measurements can predict the existence of a protective effect against infection with *B. microti*.

Mean thymidine uptake (DPM) and mean stimulation indices showed specific stimulation

---

### TABLE 1. Prechallenge uptake of $[^{3}H]$thymidine by splenocytes from immunized and control mice stimulated by mitogens and *B. microti* parasite and control antigens

<table>
<thead>
<tr>
<th>Stimulant</th>
<th>DPM (SI)$^a$ in mice with following treatment:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>None (control)</td>
</tr>
<tr>
<td>PHA</td>
<td>90 ± 13 (19.5 ± 2.0)</td>
</tr>
<tr>
<td>ConA</td>
<td>121 ± 12 (37.2 ± 7.6)</td>
</tr>
<tr>
<td>LPS</td>
<td>125 ± 21 (19.0 ± 2.2)</td>
</tr>
<tr>
<td>IRBC</td>
<td>6 ± 2 (1.0 ± 0.1)</td>
</tr>
<tr>
<td>N-RBC$^d$</td>
<td>6 ± 1 (1.1 ± 0.1)</td>
</tr>
</tbody>
</table>

$^a$ Mean DPM ± standard error of the mean $\times 10^3$ and mean stimulation indices (SI) ± standard error of the mean.

$^b$ $P < 0.02$ compared to control group.

$^c$ $P < 0.05$ compared to control group.

$^d$ N-RBC, Normal murine erythrocytes.

---

### TABLE 2. *B. microti* antibody titers and hematological values of immunized and control mice before challenge

<table>
<thead>
<tr>
<th>Immunization group</th>
<th>Mean (range) antibody titer</th>
<th>No. of nucleated cells per spleen$^a$</th>
<th>Hematocrit$^b$</th>
<th>Leukocyte count$^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>&lt;1:16</td>
<td>124 ± 9</td>
<td>36.7 ± 2.7</td>
<td>6.2 ± 1.2</td>
</tr>
<tr>
<td>Glucan</td>
<td>&lt;1:16</td>
<td>132 ± 11</td>
<td>31.0 ± 2.7</td>
<td>5.5 ± 1.4</td>
</tr>
<tr>
<td>IRBC</td>
<td>1:256 (1:128–1:512)</td>
<td>130 ± 8</td>
<td>32.7 ± 2.6</td>
<td>6.0 ± 1.3</td>
</tr>
<tr>
<td>G-IRBC</td>
<td>1:256 (1:128–1:1024)</td>
<td>142 ± 19</td>
<td>31.7 ± 2.4</td>
<td>5.4 ± 1.4</td>
</tr>
</tbody>
</table>

$^a$ Mean ± standard deviation $\times 10^6$.

$^b$ Mean ± standard deviation.

$^c$ Mean ± standard deviation per cubic millimeter $\times 10^3$. 
by the parasite or parasite-related antigen in cells from mice in the IRBC and G-IRBC groups, whereas little or no stimulation was observed in control mice or those inoculated with glucan alone. The specificity of the cellular response of the mice from the IRBC and G-IRBC groups to the presence of homologous infected RBC as antigen may be due to a parasite moiety or to an altered self antigen displayed on the surfaces of the RBC. Such a surface antigen has been detected previously in Babesia rodhaini (14); we have obtained evidence that B. microti-infected mice produce antibodies directly to antigens present on bromelain-treated homologous RBC in the hemolytic plaque assay system (G. S. Habicht and J. L. Benach, unpublished data). Similar results have been obtained in malarious mice (21). Increased \(^{3}H\)thymidine uptake in antigen-stimulated cultures by the splenocytes of immunized mice were inversely correlated with decreased parasitemia, suggesting that these in vitro responses may predict in vivo protection.

The degree of protection may be indicated by the greater antigen-specific responses obtained in the mice from the G-IRBC group when compared to the mice from the IRBC group, as evidenced by the lower mean parasitemias in the mice from the G-IRBC group. However, antibody titers were not a good indicator of the degree of protection, since mice from both the G-IRBC and IRBC groups developed similar mean titers despite the differences seen in the mean parasitemias.

Blastogenic responses to nonspecific mitogens showed no obvious functional abnormalities, except for a significant decrease in responsiveness to PHA in the splenocytes from mice of the G-IRBC group and a significantly increased response to ConA in glucan-treated mice. Cook et al. (7), using two glucan injection protocols and different time intervals between glucan injection and sacrifice, showed varied effects of glucan on murine splenocyte responses to ConA and LPS.

Unlike BCG and Propionibacterium acnes, which induce a nonspecific protective effect against challenge with low doses of B. microti (4, 5), glucan treatment alone had only a slight protective effect against subsequent challenge.

Intravenous or intraperitoneal pretreatment with live BCG (4) and formalinized Propionibacterium acnes (5) resulted in negligible parasitemias after challenge with B. microti. In both instances, a nonspecific, antibody-independent soluble mediator was thought to be responsible for the intraerythrocytic death of the parasites. Although our timing factors, dosages, and regimens of pretreatment were different, it appears that glucan by itself does not protect against B. microti as do both BCG and Propionibacterium acnes.

Evidence is now accumulating to show that glucan has diverse in vivo effects and possible modes of action. Glucan administration to mice results in increased granulocyte and macrophage production in the spleen and other sites (2), and this may account for the increased number of nucleated cells in the spleens of the treated mice. Chemically related \(\beta 1 \rightarrow 3\) glucans have been shown to alter the induction of cytotoxic T lymphocytes specific for allogeneic tumors both in vivo (10) and in vitro (11).

\(\beta(1 \rightarrow 3)\) Glucans have also been shown to activate the alternative complement pathway, resulting in an increased C3 consumption (9). This is a potentially relevant activity, since recent studies have shown that C3 and C3b receptors are necessary for in vivo development of B. rodhaini infection in rats (15). Although depletion of C3 levels in rats to below 5% of the normal levels was needed to delay the onset of parasitemia, it may be that enough C3 is consumed to retard parasite penetration of the RBC, thus rendering the organisms more vulnerable to other protective mechanisms. The in vivo effect of glucan on complement, if any, cannot be its only mechanism of adjuvanticity, since complement is not required for infection of either human or rodent RBC with Plasmodium (24); however, glucan was an effective adjuvant in a rodent-P. berghei vaccine (13).

Limited clinical trials in cancer patients have shown that glucan is relatively nontoxic, occasionally producing temporary fever and local tenderness at the site and time of the injection (18). The well-defined chemical structure of glucan, its lack of obvious toxicity, and the possibility of diverse and broad-spectrum adjuvant effects make this fungal polysaccharide of interest for further study.

ACKNOWLEDGMENTS

We are grateful to Mary Ruebush for supplying us with the mouse-adapted B. microti strain used in this study. The technical assistance of James L. Coleman and Barbara Gocinski is appreciated.

This work was supported by New York State Health Research Council grant no. 1746 and by Public Health Service grant AG00801 from the National Institute on Aging.

LITERATURE CITED
GLUCAN AS ADJUVANT FOR BABESIA IMMUNIZATION


