Fig. 4 (left). Undecagold-labeled Fab′ fragments are shown in (**A**). The Fab′ molecules show quantitative labeling and the undecagold spots (0.8 nm in diameter) are at one end of these 50-kD fragments. Some Fab′ fragments show more than one Au_{11} cluster attached. Three clear fragments, each with one gold cluster attached, can be seen in the boxed region. This vicinity is enlarged two times in (**B**); a simple schematic is shown in (**C**). Typical ferritin molecules with gold-labeled Fab′ fragments attached are shown in (**D**) and (**E**). The binding of a single Fab′-Au_{11} complex to a ferritin molecule is shown in (**F**) for clarity. Controls are shown in (**G** to **I**): Rabbit antibody to dynein Fab′-Au_{11} molecules (not shown) was prepared and appeared to be similar to (A) and was reactive toward dynein. When this Fab′-Au_{11} was incubated with ferritin and passed through a column, only native ferritin was found (G). Activated and unactivated gold clusters (with a maleimide or an amine group at the end of the linking arm) were separately incubated with ferritin, then separated on a column. Images similar to (G) were obtained, which indicated that no gold bound to the ferritin. Spectral absorbance of the gold was also used to determine that no gold had bound. Antibody to ferritin Fab′ (with no gold attached, but with the free sulfhydryl blocked with *N*-ethyl maleimide) was incubated in excess with ferritin. A subsequent incubation with antibody to ferritin Fab′-Au_{11} (and column separation) showed no gold cluster spots on the ferritin since all epitopes had been exhausted. The unlabeled Fab′ around the ferritin is clearly seen in (H) as an additional shell of protein density. When antibody to ferritin Fab′-Au_{11} was incubated with tobacco mosaic virus (TMV), glutamine synthetase (GS), and ferritin, only the ferritin was labeled (I). TMV is the rod-shaped object; GS molecules are the round, medium-intensity objects about the same diameter as TMV. Two ferritin molecules are at the lower right with dense cores and have several Fab′-Au_{11} labels attached. All incubations were for 1 hour at 37°C. Images were recorded with the STEM in the dark-field mode at a dose of 0.60 e nm^{-2}. Full width: 106 nm (A and I); 53 nm (B to H).

thin sections or in conventional EMs without a sacrifice of the high resolution of this technique.

Other antibodies have been successfully labeled by this technique and include: rabbit antibody to flagella, rabbit antibodies to 22*S* dynein and 14*S* dynein, and two mouse monoclonal antibodies to bovine factor V. This indicates that the labeling procedure described should be generally applicable to many antibodies.

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Efficacy of Murine Malaria Sporozoite Vaccines: Implications for Human Vaccine Development

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As part of a study of potential vaccines against malaria, the protective efficacy of sporozoite subunit vaccines was determined by using the *Plasmodium berghei* murine malaria model. Mice were immunized with recombinant DNA–produced or synthetic peptide-carrier subunit vaccines derived from the repetitive epitopes of the *Plasmodium berghei* circumsporozoite gene, or with radiation-attenuated sporozoites. Immunization with subunit vaccines elicited humoral responses that were equivalent to or greater than those elicited by irradiated sporozoites, yet the protection against sporozoite challenge induced by either of the subunit vaccines was far less than that achieved by immunization with attenuated sporozoites. Passive and adoptive transfer studies demonstrated that subunit vaccines elicited predominantly antibody-mediated protection that was easily overcome whereas irradiated sporozoites induced potent cell-mediated immunity that protected against high challenge doses of sporozoites. These studies indicate that new strategies designed to induce cellular immunity will be required for efficacious sporozoite vaccines.

IMMUNIZATION WITH SPOROZOITES attenuated by irradiation protects animals and humans from experimental sporozoite-induced malaria (*1*). This finding, first with *Plasmodium berghei* in rodents and subsequently with the human malaria species *P. falciparum* and *P. vivax*, provided great impetus to the development of human sporozoite vaccines. The characterization of sporozoite membrane antigens that play a role in the development of this immunity revealed a family of circumsporozoite (CS) proteins that have a general structure common to all malaria species studied to date (*2*). The gene for the CS protein of *P. falciparum*, for example, contains a long stretch of nucleotide base pairs that are highly conserved among different strains of the parasite (*3*) and encode a large central immunodominant repeat region with the amino acid sequence Asn-Ala-Asn-Pro (*4*). Antibodies elicited by immunization with synthetic peptides derived from this repeat region neutralize sporozoite infectivity, but antibodies elicited by immunization with synthetic peptides derived from two nonrepeat regions that are highly conserved among malaria species do not (*5*). The CS protein repeat sequences have thus been identified as the major target for malaria vaccine development.

There is considerable evidence that antibody to the CS protein has a role in sporozoite-induced immunity. Passive transfer of

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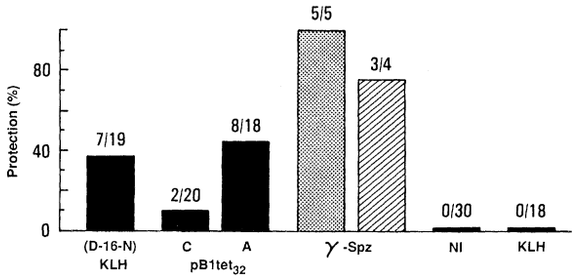

Fig. 1. Efficacy of subunit sporozoite vaccines. Mice were challenged by intravenous inoculations of 500 (■), 1,000 (▨), or 10,000 (▧) salivary gland sporozoites 2 to 4 weeks after the final immunizing dose (see Table 1). Peripheral blood smears were examined for parasitized erythrocytes daily for at least 1 month after challenge. Values above each histogram indicate the number of mice protected out of the number challenged; protected animals failed to develop patent parasitemia during the follow-up period. Immunizing antigens are indicated under each histogram. Construct $pB1tet_{32}$ was combined with either complete Freund's adjuvant (C) or alum (A); NI, nonimmunized mice; γ-Spz, mice immunized with γ-irradiated sporozoites.

monoclonal antibodies (mAb) to *P. berghei* CS protein protects recipient animals (*6*), and humans living in endemic areas have CS antibodies with biologic activities that correlate with protection from sporozoite challenge (*7*). Subunit vaccines prepared against *P. falciparum* CS protein have been developed both by recombinant DNA techniques and by synthesizing peptides and are being tested for safety and immunogenicity in human volunteers, but their efficacy has not been tested by challenge with viable sporozoites. The CS gene for *P. berghei* has recently been cloned and sequenced (*8*), making it possible to use the rodent malaria model for testing sporozoite vaccines. In the study reported here we used this murine model to demonstrate that immunization with subunit CS protein vaccines induces high levels of antibodies to *P. berghei* sporozoites, but that the protection obtained is quantitatively and qualitatively different from that induced by immunization with irradiated sporozoites. The data suggest that vaccines that also elicit cell-mediated immunity will be required for human antisporozoite vaccines.

A 16-residue peptide (D-16-N) containing two tandemly repeated octapeptides from the repeat region of the *P. berghei* CS protein was synthesized and conjugated to keyhole limpet hemocyanin (KLH) (*9*). A *P. berghei* CS construct, $pB1tet_{32}$, was expressed in *Escherichia coli* as a fusion protein containing 254 amino acids including the entire central repeat region of the CS protein (*10, 11*). C57Bl/6 mice were immunized with (D-16-N)-KLH or $pB1tet_{32}$, with complete Freund's adjuvant (CFA) or alum being used as adjuvants (*12*) (Table 1). Control mice were immunized with 50 μg of KLH in CFA or with multiple intravenous doses of gamma-irradiated (γ) *P. berghei* sporozoites (*13*).

Sera from the mice immunized with (D-16-N)-KLH, $pB1tet_{32}$, or γ-sporozoite–immunized mice contained high titers of antibodies that reacted in an enzyme-linked immunosorbent assay (ELISA) (*14*) when (D-16-N) conjugated to bovine serum albumin [(D-16-N)-BSA], $pB1tet_{32}$ or *P. berghei* sporozoites were used as antigens (Table 1). Sera from these mice also blocked sporozoite invasion of human hepatoma cells in vitro (ISI assay) and reacted in the circumsporozoite precipitation (CSP) assay (*14*); both assays are thought to correlate with protection from sporozoite challenge. Immunization with CS subunit vaccines induced antibodies with higher ELISA titers and greater CSP and ISI activities than did immunization with γ-sporozoites.

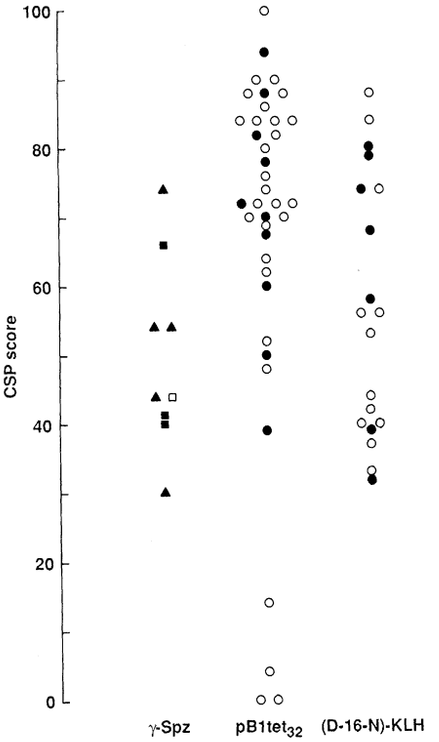

Fig. 2. Relation between CSP scores and protection from sporozoite challenge. CSP assays were performed as described (*14*) with sera diluted 1:2. Each of 25 sporozoites was scored as 0 (no reaction), 2 (surface precipitate), or 4 (filamentous precipitate), and the sum of these scores was plotted for individual mice immunized with irradiated sporozoites (γ-Spz), recombinant DNA construct ($pB1tet_{32}$), or synthetic peptide conjugate (D-16-N)-KLH. Mice were challenged intravenously with 500 (○), 1,000 (△), or 10,000 (□) NK65 *P. berghei* sporozoites. Solid symbols indicate protected mice. All animals challenges with the 1,000-sporozoite dose were protected.

Table 1. Immunization schedule and serum reactivity. ND, not done.

Immunogen*	Dose	Schedule (weeks)	ELISA titer†			CSP‡ score	ISI§
			(D-16-N) BSA	$pB1tet_{32}$	NK65		
(D-16-N)-KLH-CFA	100 μg	0,3,7,12	30,800	10,580	2,250	84	93
$pB1tet_{32}$-CFA	50 μg	0,2,4,8	3,600	23,295	6,150	89	97
$pB1tet_{32}$-alum	50 μg	0,4,8	2,975	12,475	4,063	83	94
γ-Sporozoites	50,000	0					
	11,000	2	3,000	3,000	3,940	62	85
	2,000	4					
KLH-CFA	50 μg	0,3,7,12	0	0	0	2	ND

*Structure of synthetic peptides and recombinant construct: (D-16-N) = (DPAPPNANDPAPPNAN); $pB1tet_{32}$ = MDRTPS(1-254)DPtet_{32}. †Titer determined by ELISA with (D-16-N)-BSA, $pB1tet_{32}$, or NK65 sporozoites being used as antigen. At each dilution the standard deviation was less than 0.1 optical density (O.D.) unit. The geometric mean titers for grouped sera were calculated by using the dilution of individual sera, giving an O.D. of 1 (50% maximal absorbance), and were reproducible to within a single dilution of serum. ‡Circumsporozoite precipitation score (see legend to Fig. 2). §Inhibition of sporozoite invasion into HepG16-2A cells in vitro by serum diluted 1:20 (*14*). Both CSP scores and ISI activities were reproducible to within 5%.

Protective efficacy of these vaccines was determined by intravenous challenge with 500 to 10,000 sporozoites from the salivary glands of mosquitoes (*Anopheles stephensi*). When all groups of mice that received the subunit vaccine were considered, only 17 out of 57 (30%) were protected from challenge with 500 sporozoites (Fig. 1). None were protected when challenged with 1000 sporozoites. The mice that received their first three doses of $pB1tet_{32}$-CFA at 2-week intervals appeared to have decreased protection. In no subunit-vaccinated group did protection exceed 45%. In contrast, all but one of the animals immunized with γ-sporozoites were protected even when challenged with a 2- to 20-fold greater dose of sporozoites. Protection of subunit-vaccinated mice was never seen in the absence of detectable antibody, but high levels of CSP activity did not assure protection in these groups (Fig. 2). In contrast, γ-sporozoite–immunized mice had CSP activities comparable to subunit-vaccinated individuals, yet resisted

much greater sporozoite challenges. Analyses of ISI and ELISA results for individual animals were similarly not predictive of protection.

In previous studies, passive transfer of serum from sporozoite-immunized animals did not protect recipient animals from challenge, but passive transfer of purified mAb to a repetitive epitope on *P. berghei* sporozoites did protect recipient mice in a dose-dependent manner (*6, 15*). To determine whether protection induced by subunit vaccines was a function of antibody concentration, we used a protein A column to purify immunoglobulin G (IgG) from serum obtained from the mice that received (D-16-N)-KLH, pB1tet$_{32}$, or γ-sporozoites and were protected when challenged with 500 sporozoites (*16*). *Plasmodium berghei* mAb 3.28 (IgG1), which reacted with CS protein by immunoblot and produced high ELISA, CSP, and ISI activity was similarly purified. These antibodies were injected intravenously into naïve mice 30 minutes before they were challenged with 500 sporozoites. As shown in Table 2, mAb 3.28 protected recipient animals in a dose-dependent fashion, with 100% protection in all mice receiving at least 100 μg of mAb. Protection with polyclonal serum was observed only with the 250-μg dose of (D-16-N)-KLH IgG, which contained 5.65 μg of CS-specific antibody (2.26% of 250 μg). These data suggest that polyclonal antibodies in mice injected with CS subunit vaccines may be more effective than monoclonal antibodies. However, levels of CS antibodies produced with γ-sporozoite immunization were comparable to those elicited by subunit vaccination yet γ-sporozoite immunized mice resisted challenge with at least 20 times more sporozoites than subunit vaccinated mice. These data indicate that the mechanism of protective immunity elicited by γ-sporozoites is fundamentally different from that induced by immunization with subunit CS protein vaccines and may be independent of antibody.

The potential importance of antibody-independent mechanisms was first suggested by experiments with sporozoite-immunized mice that had been treated with goat antibody to the μ chain of immunoglobulin M and therefore could not produce antibody but were protected against sporozoite challenge (*17*). Adoptive transfer experiments by one of us (W.T.H.; see Fig. 3A) showed that T-cell–enriched spleen cells from γ-sporozoite–immunized donors protected recipient animals, but B-cell–enriched spleen cells did not, extending an earlier observation that sensitized whole spleen cell populations conferred protection (*18*). We similarly examined the development of cellular

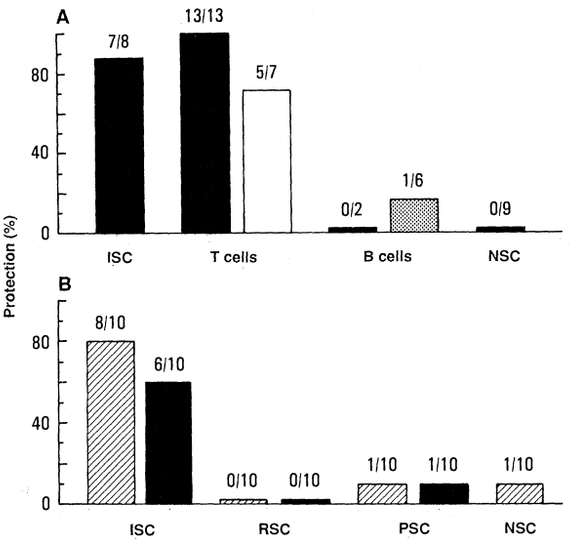

Fig. 3. Adoptive transfer of immunity to sporozoite challenge. (**A**) Recipient mice received 2.5 × 10^6 (■), 1 × 10^6 (□), or 5 × 10^5(▩) spleen cells from mice immunized with γ-sporozoites. Immune spleen cells (ISC) were fractionated by nylon wool columns. Nonadherent cells were treated with antisera to light chains of immunoglobulin to yield a population enriched for T lymphocytes. Adherent cells were treated with antisera to theta chains of immunoglobulin to yield a population enriched for B lymphocytes. NSC, normal spleen cells. (**B**) Recipient mice received 1 × 10^7(▧) or 2.5 × 10^6 (■) unfractionated spleen cells from mice that were protected following immunization with the recombinant protein pB1tet$_{32}$-alum (RSC) or the synthetic peptide (D-16-N)-KLH-CFA (PSC). Control mice received total spleen cell populations from γ-sporozoite immunized (ISC) or nonimmune (NSC) mice. Recipient mice were irradiated with 500 R immediately prior to transfer and received a booster injection of 10,000 γ-sporozoites 24 hours later. Mice were challenged with 10,000 (A) or 1,000 (B) salivary gland sporozoites 7 days after cell transfer. The number that did not develop patent parasitemia out of the number challenged is shown for each group.

Table 2. Protective efficacy of passively transferred IgG purified from ascites containing mAb 3.28 or from sera of normal mice (control) or mice that were immunized with (D-16-N)-KLH, pB1tet$_{32}$, or γ-sporozoites and shown to be protected from sporozoite challenge (*15*). The concentration of antigen-specific IgG was determined by ELISA with (D-16-N)-BSA as the capture antigen and expressed as percentage activity relative to mAb 3.28. Data are expressed as the number of mice protected out of the number challenged. The mean day that parasitemia was first detected is given in parentheses. ND, not done.

Dose (μg)	IgG source (and percentage activity)				
	mAb 3.28 (100%)	(D-16-N)-KLH (2.26%)	pB1tet$_{32}$ (1.05%)	γ-Sporozoites (0.03%)	Control (0%)
500	4/4	ND	ND	ND	ND
250	4/4	3/4 (7.0)	0/4 (6.5)	0/4 (5.8)	0/4 (5.8)
100	4/4	0/3 (5.7)*	0/4 (5.0)*	ND	ND
50	1/4 (5.3)	ND	ND	0/4 (5.7)	0/4 (4.8)
25	0/4 (5.8)	0/4 (5.8)	0/4 (5.7)	0/4 (5.0)	0/4 (4.8)

*The dose was 125 μg for these groups.

immune responses in mice vaccinated with subunit CS protein, contrasting these responses with those elicited by γ-sporozoite immunization. Sensitized spleen cells from mice immunized with and protected by γ-sporozoites, (D-16-N)-KLH-CFA, or pB1tet$_{32}$-alum were adoptively transferred into naïve animals that received boosting doses (10,000 γ-sporozoites) 24 hours later and were challenged after 1 week with 1000 sporozoites (Fig. 3B). Spleen cells from sporozoite-immunized donors, but not from subunit vaccinated donors, protected the recipient mice.

These studies have important implications for sporozoite malaria vaccine development. It may be possible to improve the efficacy of subunit vaccines by modification of the immunization regimen or dose (*19*), alteration of the orientation of the peptide-carrier coupling (*20*), inclusion of other sporozoite-derived epitopes, or the use of more potent adjuvants. In this study we also challenged mice with intravenous injections of sporozoites. Under natural conditions animals are challenged by mosquito bites so that sporozoites are introduced into capillaries and are likely to be exposed longer to the protective effects of antibody prior to reaching the liver. These studies differentiated between cellular and humoral protective responses induced by vaccination and emphasize that cellular immune mechanisms are critical to the high level of protection induced by γ-sporozoites. The correlation of protective immunity with CS antibody as indicated by CSP and ISI activity in animal and human immunization studies may largely be an epiphenomenon marking induction of critical but unrecognized cell-mediated immunity. In theory, animals vaccinated with peptide-carrier conjugates preferentially induce humoral responses since T epitopes required for cellular responses (excluding T-helper

activities) are generally not found on short synthetic peptides. Unlike the repeat region of *P. falciparum*, the *P. berghei* CS protein contains variable tandem repeats consisting of three different octapeptides and a long stretch of dipeptides (*8*). We used the consensus octapeptide for synthetic peptide immunization, but it is possible that other epitopes may be important in protection. The recombinant construct pB1tet$_{32}$ included the entire repeat region sequence as well as flanking nonrepeat regions, suggesting that if T-cell epitopes involved in the induction of cellular immunity were included they were ineffective because of inappropriate conformation or antigen presentation. Attenuated sporozoites must be intact and administered intravenously to be efficacious (*21*), suggesting that targeting of the organism to a particular cell and subsequent processing and presentation of antigen in association with histocompatibility molecules may be important. From studies in vitro it appears that sporozoites shed CS protein onto the surface of hepatoma cells during invasion and that developing exoerythrocytic forms express epitopes recognized by mAbs against sporozoites (*22*). It is possible that CS protein or other non-CS antigens associated with these developing exoerythrocytic forms elicit responses mediated by natural killer cells, cytotoxic T cells, or cytokines. However, the antigens, specific cellular effector mechanisms, and their targets have not been identified. By using the *P. berghei* model it should be possible to develop subunit vaccines capable of inducing these cell-mediated responses and to apply these findings to the development of sporozoite vaccines against human malaria.

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9. Peptides were synthesized by the solid phase technique of R. B. Merrifield *et al.* [*Annu. Rev. Biochem.* **39**, 841 (1970)] with a Beckman 990 peptide synthesizer. Purity was confirmed by reversed-phase high-performance liquid chromatography and amino acid composition analysis. Equal amounts of peptide and KLH were combined (1:1 w/w) in 1% glutaraldehyde in phosphate-buffered saline (PBS) and stirred for 3 hours at room temperature, dialyzed extensively against PBS, and lyophilized to dryness.
10. A Taq I fragment encoding 82% of the complete *P. berghei* CS gene was modified to prepare clone pB1-6 (J. L. Weber *et al.*, *Exp. Parasitol.*, in press), digested with Taq I, treated with DNA polymerase I (Klenow fragment) and deoxycytidine triphosphate to partially fill in the 5′ overhang, and treated with mung bean nuclease to remove the remaining single base overhang. The DNA was digested with Ssp I and a 763–base pair fragment was isolated and ligated into Bam HI–digested Klenow-treated pAS1 that had been modified by deleting a 14–base pair Bam II fragment in the tet^r regions as described [Young *et al.* (*11*)]. The resulting construct encodes 254 amino acids and extends from Taq I, which begins 40 amino acids 5′ of the first octapeptide repeat, includes all of region I (*4*), the central repeat units, region II (*4*), and ends in the hydrophobic anchor sequence 18 amino acids 5′ of the carboxyl-terminal asparagine residue of the complete protein. This polypeptide is fused to 32 amino acids derived from the tet^r region of the expression vector. The construct was expressed in *E. coli* under the control of the P_L promoter as described [J. F. Young, *Proc. Natl. Acad. Sci. U.S.A.* **80**, 6105 (1983)]. The *P. berghei* fusion protein extracted from induced *E. coli* with 0.1% (w/v) sodium deoxycholate was present at approximately 5 to 10% of the total protein precipitated with 20 to 40% ammonium sulfate.
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12. Antigens were emulsified 1:1 in complete Freund's adjuvant [CFA], (Gibco) for primary immunization and in incomplete Freund's adjuvant (IFA) for subsequent doses, and injected intramuscularly and subcutaneously. CS construct pB1tet$_{32}$ was adsorbed to aluminum hydroxide gel in 0.9*N* saline by end-over-end rotation at 4°C for 16 hours. Each 0.25-ml dose contained 0.5 mg of Al^{3+}.
13. Sporozoites of the NK65 strain of *P. berghei* were harvested in Medium 199 from *Anopheles stephensi* approximately 14 days after the mosquitoes had fed on infected Syrian hamsters. Sporozoites used for immunization received 8000 R from a ^{60}Co source (Gamma Cell 220) at 4°C.
14. The ELISA, ISI, and CSP assays were performed under blind conditions on triplicate serum samples as described by Young (*11*) with the following modifications: (i) the capture antigen for ELISA was (D-16-N)-BSA, pB1tet$_{32}$, or *P. berghei* sporozoites (NK65 strain); (ii) for the ISI and CSP assays *P. berghei* sporozoites were used.
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16. Pooled serum samples from mice immunized with γ-sporozoites (D-16-N), pB1tet$_{32}$, and from normal controls (20 mice per group), and mAb 3.28.1 ascites were affinity-purified by protein A–Sepharose 4B chromatography (*7*). The mAb 3.28.1 was derived from spleen cells of mice immunized with γ-sporozoites as described elsewhere [H. D. Danforth, G. H. Campbell, M. F. Leef, R. L. Beaudoin, *J. Parasitol.* **68**, 1029 (1982)]. The protein concentration of the final product was determined spectrophotometrically at a wavelength of 280 nm.
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Glucocorticoid Receptor–Like Antigen in Lymphoma Cell Membranes: Correlation to Cell Lysis

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S-49 mouse lymphoma cells undergo lysis when treated with glucocorticoids; the mechanism of this effect is not understood. A protein was detected in the plasma membrane of these cells by means of direct immunofluorescent labeling with a monoclonal antibody to the soluble glucocorticoid receptor. Cellular heterogeneity in the content of this glucocorticoid receptor–like molecule was evident. By immunoadsorption to antibody-coated tissue culture plates, the cells were separated into populations positive (100%) and depleted (38%) for this membrane antigen. Gel electrophoresis, specific immunoblot, and autoradiographic (binding of [^{3}H]dexamethasone mesylate) analysis of the membrane proteins from the membrane antigen–positive group revealed multiple protein bands ranging in size from 85 to 145 kilodaltons. Furthermore, comparison of the glucocorticoid sensitivity of these groups of cells showed complete lysis of the membrane antigen–positive cells and only partial lysis of the antigen-deficient group, which suggests that the lysis response of cells to glucocorticoids is mediated by a glucocorticoid receptor–like molecule located in the plasma membrane.

GLUCOCORTICOID HORMONES ELICIT a series of cellular responses in lymphoid tissues that ultimately result in cytolysis, a property that allows important practical applications of these steroids in the treatment of certain leukemias and lymphomas (*1, 2*). Although the exact mechanism of this induced cell lysis is obscure, the effect is known to be mediated by the glucocorticoid receptor (GR) (*3*). In some patients with various blood dyscrasias, high numbers of lymphoid cell receptor sites have been correlated with good clinical response to gluco-

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