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# Survival and Antigenic Profile of Irradiated Malarial Sporozoites in Infected Liver Cells

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**Exoerythrocytic (EE) stages of *Plasmodium berghei* derived from irradiated sporozoites were cultured in vitro in HepG2 cells. They synthesized several antigens, predominantly but not exclusively those expressed by normal early erythrocytic schizonts. After invasion, over half the intracellular sporozoites, both normal and irradiated, appeared to die. After 24 h, in marked contrast to the normal parasites, EE parasites derived from irradiated sporozoites continued to break open, shedding their antigens into the cytoplasm of the infected host cells. Increasing radiation dosage, which has previously been shown to reduce the ability of irradiated sporozoites to protect animals, correlated with reduced de novo antigen synthesis by EE parasites derived from irradiated sporozoites.**

In 1967 Nussenzweig et al. (29) reported that mice could be protected against sporozoite-induced malaria if they were previously immunized with X-irradiated sporozoites. This phenomenon was subsequently confirmed for human plasmodial species (5). The mechanism of protection was thought to be mediated primarily by antibodies directed against the circumsporozoite proteins (CSP). Although such antibodies can neutralize sporozoite infectivity (20, 26) and high anti-CSP titers can protect humans against a sporozoite challenge (16), it is now thought that a major component of the protective response generated by irradiated sporozoite is mediated by $CD8^+$ (cytotoxic) T cells directed at the liver or exoerythrocytic (EE) stage (17, 33, 47). Intrahepatic CSP (20, 39) is thought to be processed (44) by the hepatocyte and presented as a target for protective $CD8^+$ T cells in rodents (31, 32), and circumstantial evidence suggests that this is also true in humans (18, 24).

The highly effective $CD8^+$ T-cell-based immunity achieved with irradiated sporozoites is thought to be generated by the infected hepatocyte (32, 47), since only these cells support EE development, permitting consequent endogenous presentation (27, 44) of parasite antigens to class 1 major histocompatability antigens (MHC) (17). (Whether sporozoites found in macrophages [11] can present antigens to class 1 MHC [7] and generate protective $CD8^+$ T cells [32] remains untested.)

To understand more fully the basis of the immunity generated by the EE parasites derived from irradiated sporozoites (EErads), their antigenic repertoire and survival was studied in an in vitro system with the rodent parasite *Plasmodium berghei* and the human hepatoma line HepG2 as host liver cells.

## MATERIALS AND METHODS

**Irradiation of sporozoites.** Traditionally X-ray sources were used to irradiate sporozoites (28, 45); however, recently gamma-ray sources have been employed to attenuate sporozoites (36). Both sources give comparable levels of protection (37, 47).

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In a series of initial comparative experiments, sporozoites were given 10,000 rads of X rays and the same dose of gamma rays from a $^{60}Co$ source. The radiation dose was measured with a Baldwin-Falmer dose meter, which measures the absorbed dose in air; the absorbed dose in soft tissue is 1.095 times higher. The survival characteristics and the antigens synthesized by EErads were indistinguishable for both types of radiation (data not shown). The irradiated sporozoites referred to in this report were pooled sporozoites that were dissected from mosquitoes immediately after the intact insects had received 10,000 rads (unless otherwise stated) of $^{60}Co$ irradiation (dose rate, 600 rads per min).

**Culture of EE stages.** EE and EErad parasites were cultured in HepG2 cells grown on 13-mm-diameter glass cover slips as described previously (39, 40), except that the medium was changed only once daily. For the data referred to herein we used host HepG2 cells that had received 1,700 rads of $^{60}Co$ radiation (21) 3 to 24 h before infection with sporozoites to prevent overgrowth of the culture. The use of unirradiated HepG2 cells in separate comparable experiments did not significantly alter the survival curves or antigen expression of EErads (data not shown).

**Assays for antigen expression, survival, and size of parasites.** The indirect immunofluorescent antibody test (IFAT) (39, 40) was used to count parasites with the following reagents as primary antibodies. Anti-CSP monoclonal antibody (MAb), specific for the CSP of *P. berghei* (3D11) (39, 49), reacts with the normal EE schizont of *P. berghei* throughout its 48- to 72-h intrahepatic life cycle (20, 39, 50). Anti-Pbl1 MAb recognizes a liver-specific antigen of *P. berghei*, which is believed to be localized to the parasitophorous vacuole membrane and present on all EE parasites after 3 to 5 h (A. Suhrbier, L. A. Winger, C. A. O'Dowd, K. J. Hodivala, and R. E. Sinden, Parasite Immunol., in press). Rabbit polyclonal anti-*Plasmodium falciparum* heat shock fusion protein (Pfhsp70) (4) stains *P. berghei* blood stage parasites, newly invaded sporozoites, and all subsequent EE stage parasites but not sporozoites (unpublished observation; N. Kumar et al., Bull. W.H.O., in press). W3.5 MAb was specific for a 31-kilodalton protein found in the cytoplasm of schizont-infected erythrocytes (49) and was present in EE stages after about 36 h (42). F4.4 MAb, which reacts with the precursor of the major merozoite surface

TABLE 1. Effects of irradiation[a]

| Radiation dose (rads) | % Invasion | No. of parasites at 24 h | | % of Pbl1+ EErads |
|---|---|---|---|---|
| | | CSP+ | Pbl1+ | |
| 0 | 20.9 ± 11.4 | 3,517 ± 285 | 2,975 ± 784 | 85 |
| 10,000 | 22.0 ± 6.0 | 3,436 ± 14 | 2,436 ± 127 | 70 |
| 0 | 19.6 ± 3.0 | 1,257 ± 1,477 | 1,151 ± 212 | 91 |
| 15,000 | 15.1 ± 2.4 | 2,882 ± 499 | 825 ± 141 | 28 |
| 0 | 31.9 ± 0.56 | 3,447 ± 1,014 | 2,462 ± 213 | 71 |
| 50,000 | 34.9 ± 8.8 | 2,723 ± 542 | 187 ± 67 | 7 |

[a] The effects of radiation on invasion rates and numbers of parasites on a series of parallel cover slips detected at 24 h with anti-CSP MAb and anti-Pbl1 MAb in the IFAT. The percentage of parasites that had synthesized Pbl1 at 24 h postinvasion was determined with the formula: (number of Pbl1-positive parasites at 24 h)/(number of CSP-positive parasites at 24 h) × 100. Each radiation dose experiment was performed with separate mosquito batches.

antigen (PMMSA) of *P. berghei*, reacts with all EE parasites after 36 to 40 h (40, 48).

All parasite numbers given refer to the total number of parasites found per 13-mm cover slip, expressed as an average of three replicate preparations. Where several antibodies and time points were used in one experiment, a series of parallel cover slips was set up with three cover slips fixed for each time point and antibody. The data given for each figure or table are from a single experiment and are representative of three repeated experiments (except Table 1, in which the experiment was repeated twice).

Anti-Pbl1 was used to measure parasite size because it stains the periphery of the parasite. It is thought to locate to the parasitophorous vacuole membrane (Suhrbier et al., in press).

**DAPI staining.** To examine the effect of irradiation on nuclear replication, EE parasites and EErads were stained with 4,6-diamidino-2-phenylindole (DAPI; Sigma, United Kingdom) as described previously (22), except that the cultures were not dried.

## RESULTS

**Invasion and survival over the first 24 h.** Irradiated and normal sporozoites from the same mosquito infection were added separately to triplicate cultures, and the numbers of intracellular parasites were determined by using anti-CSP MAb in the IFAT. The problem of counting sporozoites that had not invaded but were merely adhering to the surface of host cells (30) was overcome by repeated washing of the cover slips with medium 1 h after the addition of the sporozoites. As judged by using the method of Renia et al. (30), which distinguishes between intra- and extracellular parasites, this procedure removed 80 to 90% of uninvaded sporozoites (unpublished observation). Radiation did not affect the sporozoite invasion rate (Table 1). The survival of EErads (dose, 10,000 rads) and EE parasites derived from normal sporozoites up to 24 h was also indistinguishable. Approximately 40 to 45% of sporozoites that invaded after 1 h survived for 24 h in both cases. In one experiment, $2.5 \times 10^4$ sporzoites were added per cover slip. After 1 h (using anti-CSP MAb in the IFAT), 4,800 ± 1,772 parasites were detected in cultures infected with normal sporozoites and 4,152 ± 2,260 parasites were detected in cultures infected with irradiated sporozoites. After 24 h, these values had fallen to 1,944 ± 556 and 1,888 ± 668, respectively (see also Fig. 3). (This survival is subject to large variation between experiments [unpublished observation].)

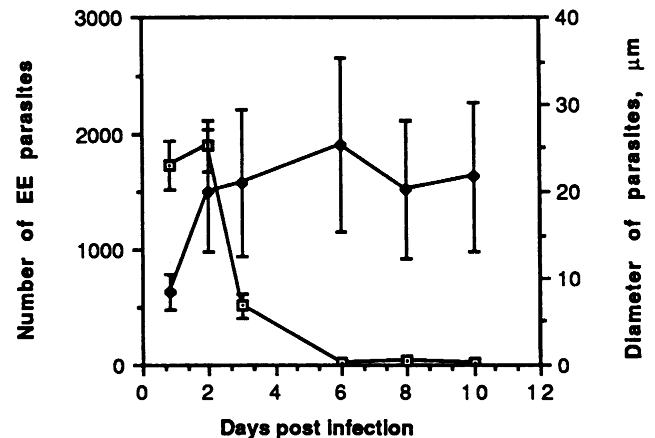


FIG. 1. The numbers (□) and diameters (◆) of EE parasites derived from normal sporozoites ($2.4 \times 10^4$ per cover slip) grown in HepG2 cells over 10 days. Bars represent standard deviations. Parasites were detected and measured by using anti-Pbl1 MAb and the IFAT.

**Survival and size after 24 h.** In two separate experiments (with different mosquito infections) the numbers and sizes of EErads (Fig. 1) and normal EE parasites (Fig. 2) were determined from 24 h to 10 days by using anti-Pbl1 in the IFAT. (The shapes of the survival curves in Fig. 1 and 2 were not dependent on the type of antibody used; the survival data were not significantly different when anti-CSP MAb was used in separate comparable experiments [data not shown].)

The number of EE parasites that synthesized Pbl1 derived from normal sporozoites did not fall between 24 and 48 h, and as the parasites completed the liver phase of development (48 to 72 h) parasite numbers decreased rapidly as they detached from the monolayer as mature segmenters (20, 41). Parasites derived from normal sporozoites increased in size dramatically between 24 and 48 h, after which they no longer increased significantly in size, leaving a small number of large parasites that survived for the full 10-day experimental period.

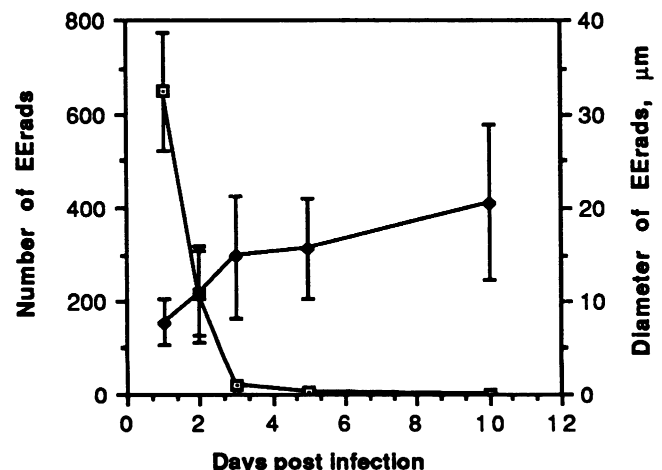


FIG. 2. The numbers (□) and diameters (◆) of EE parasites derived from irradiated (dose, 10,000 rads) sporozoites ($1.34 \times 10^4$ per cover slip) grown in HepG2 cells over 10 days. Bars represent standard deviations. Parasites were detected and measured by using anti-Pbl1 MAb and the IFAT.

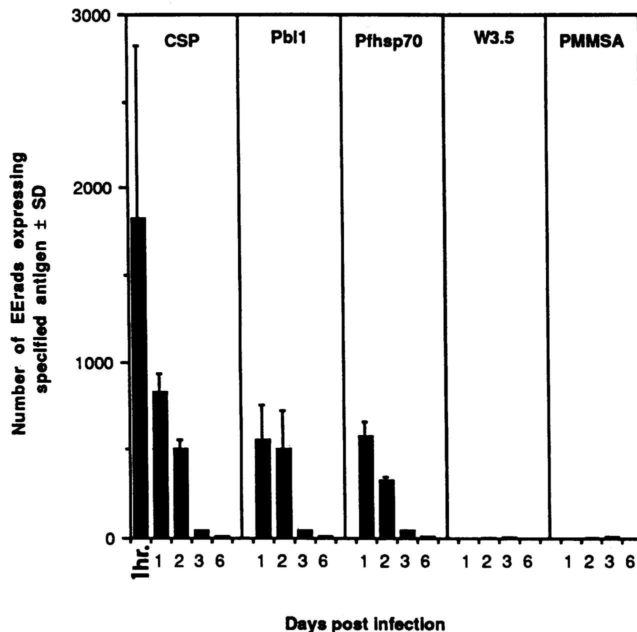


FIG. 3. Expression of five selected antigens by EE parasites derived from irradiated (dose, 10,000 rads) sporozoites ($1.8 \times 10^4$ per cover slip). Bars represent standard deviations.

EErad numbers continued to fall after 24 h, leaving only a very small number of parasites at 3 days. The EErad size increase tended to be slower over the first 3 days, after which only a slow rate of increase in size was observed in the small number of parasites, which again persisted for 10 days (Fig. 2).

**Antigen expression of EErads.** Irradiated sporozoites were added to a series of parallel cover slips and fixed 1, 2, 3, and 6 days later. The number of EErads was determined by using anti-CSP MAb 3D11 in the IFAT, and the number synthesizing any one of the four additional selected antigens was determined by IFAT in parallel cultures (Fig. 3). Antigens that are known to be expressed early in liver stage development (Pbl1, Pfhsp70) were expressed by well over half of the EErads (dose, 10,000 rads) throughout their lives; however, antigens invariably expressed later in normal development (W3.5 and PMMSA) (40, 42) were only seen in a small proportion of the older EErad population. As reported by Sigler et al. (36), polyclonal blood-stage serum, raised by repeated inoculation of mice with blood-stage parasites, also stained EErads and in our hands gave results similar to those with anti-Pfhsp70 (data not shown).

**Effect of radiation dose on antigen expression.** By using separate mosquito infections (one for each dose of radiation) the effect of the radiation dose was determined on (i) the invasion rate, measured by using anti-CSP MAb in the IFAT 1 h after the addition of sporozoites; (ii) the number of parasites observed at 24 h, counted by using anti-CSP MAb in the IFAT; (iii) the number of parasites observed at 24 h, counted by using anti-Pbl1 MAb in 3 parallel cover slips; and (iv) the percentage of 24-h parasites, measured by using anti-CSP that had synthesized Pbl1 (i.e., [iii/ii] × 100).

Radiation doses up to 50,000 rads had no effect on the invasion rate, but the EErads surviving at 24 h progressively lost their ability to synthesis Pbl1 as the radiation dose was increased (Table 1). (The survival curve of EErads that had received 50,000 rads was slightly steeper than that seen in Fig. 2, and parasites were smaller [data not shown].)

**Development of EErads.** After invasion and transformation into a trophozoite (at around 16 to 24 h), the appearance of EErads when viewed with anti-CSP MAb (39), anti-Pbl1 MAb (Suhrbier et al., in press), and anti-Pfhsp70 (Fig. 4) was similar to that of control parasites of the same age. After 24 h, however, the EErads underwent abnormal development; although an increase in size was observed, phase-contrast pictures of the parasites often revealed that the parasites only had a limited amount of cytoplasm and appeared swollen with fluid (Fig. 5). A dose of 10,000 rads did not completely prevent DNA replication of the EErads; several irregularly sized nuclei could clearly be seen in many parasites after staining with DAPI (Fig. 6). At 3 days a normal EE parasite contains several hundred merozoites (19, 20), which stain brightly with DAPI (22; A. Couchman, personal observation).

Approximately 1 in 100 to 300 infected hepatoma cells was stained throughout the cytoplasm with any given antibody. These "whole-cell positives" were apparent 1 h after invasion in both normal and irradiated sporozoites when anti-CSP MAb was used in the IFAT (Fig. 8). No such whole-cell positives were observed at any of the other time points taken (i.e., 1 to 10 days) for normal parasites. In contrast, in cultures infected with irradiated sporozoites, whole-cell positives were observed on days 1 to 6 and were also revealed on these days with the other antibodies used in this study (Fig. 7). Broadly similar numbers of these whole-cell positives were also observed with anti-CSP MAb in 1-h cultures in which sporozoites had received 15,000 or 50,000 rads.

## DISCUSSION

As reported previously (29a, 36), sporozoite invasion and transformation into trophozoites are unaffected by irradiation. Instead of the rapid growth and replication, which then occur in normal development, EErads undergo limited nuclear replication (29a, 36) and a slower increase in size, which in part appeared to be due to fluid uptake (Fig. 5). Limited in vivo observations support this general trend (45, 46). Higher doses of radiation, however, completely prevent nuclear replication (29a, 36). The amount of development of EErads has also been shown to be affected by the species of hepatocyte host cell used to culture the parasite (29a).

What is striking is the disappearance of the vast majority of EErads within the same time frame (3 days) as that in which normal EE parasites mature and leave the monolayer as segmenters (19, 41). Vanderberg et al. also reported a lack of appreciable numbers of the EErad (dose, 2,000 to 4,000 rads) form of *P. berghei* in vivo after 3 days (45). The presence of detectable numbers of EErads (dose, 6,500 to 7,500 rads) in liver biopsies from *Plasmodium cynomolgi*-infected monkeys 8 days after infection (46) further supports the notion that the survival time of the majority of EErads is very similar to the time taken to complete normal EE development, which in the case of *P. cynomolgi* is 8 to 9 days (15).

It has been suggested that EErads persist for long periods of time (3). In this in vitro analysis only a small number of persisting EErads was observed; using a similar system, Sigler et al. (36) reported a total loss of EErads (dose, 8,000 and 15,000 rads) after 6 days. Persistent parasites were also seen in this study after infection of HepG2 cells with normal sporozoites. This phenomenon also occurred when nonirradiated HepG2 host cells were used (data not shown).

Landau and Killick-Kendrick (25) have suggested that

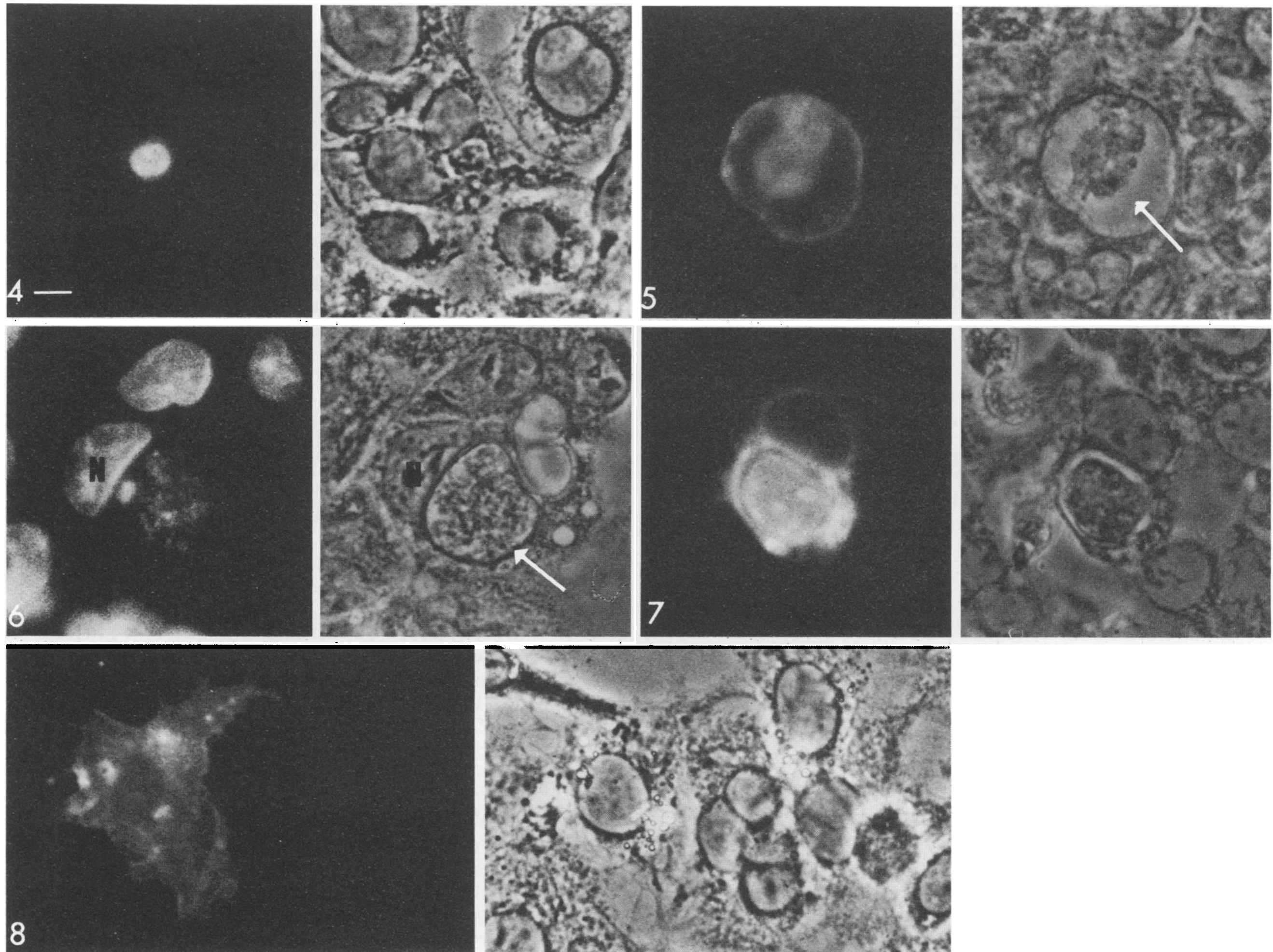


FIG. 4–8. IFAT pictures accompanied on their right by phase photographs of the same field. The magnifications for all photographs are the same. Bar = 10 μm.

FIG. 4. EErad (24 h) stained with anti-Pfhsp70.

FIG. 5. EErad (7 days) stained with a mouse polyclonal anti-blood-stage serum. In the phase picture the parasite appears swollen with fluid (arrow).

FIG. 6. DAPI staining of a 3-day culture showing staining of host cell nuclei (N) and irregular distribution of parasite DNA within the EErad (arrow on phase picture indicates the periphery of the EErad).

FIG. 7. EErad (5 days) stained with anti-PMMSA MAb showing extensive labeling of the host cell cytoplasm.

FIG. 8. HepG2 culture stained with anti-CSP MAb 1 h after infection with normal sporozoites. CSP appears to be localized throughout the cytoplasm of one cell.

small numbers of persisting multinucleate forms of *P. berghei* exist in *Thamnomys* species for up to 8 months after infection with normal sporozoites. Persisting forms unrelated to the uninucleate hypnozoite (23) are also well documented in other plasmodia (34, 35). Vanderberg et al. (45) also observed one EErad (dose, 2,000 rads) in a mouse 16 days after *P. berghei* sporozoite infection. Whether the persisting forms observed in this study represent a true parasite phenotype or an in vitro artifact and whether the very small number of persisting forms would contribute significantly to protection remain unclear.

In this in vitro system, over half of the invaded sporozoites, whether irradiated or not, appeared to die during the first 24 h of culture, shedding their antigens, notably CSP, into the host cell cytoplasm. It is unclear whether this is a feature of this species of parasite or relates to the suitability of the hepatoma host cell in vitro (6). How important such a phenomenon might be in generating immunity (3) also remains to be established. One might speculate that if this phenomenon also occurred in mice (and caused early large-scale endogenous presentation of sporozoite antigens to class 1 MHC), it would result in enhanced activation and localization of CSP-specific $CD8^+$ T cells in the livers of immunized mice very early after a sporozoite challenge. Host-parasite combinations that do not suffer from such a high postinvasion sporozoite mortality might not be so easy to protect with CSP-specific cytotoxic T cells.

In contrast to normal EE parasites, after 24 h EErads continue to disappear from the cultures. The rare appearance of the entire cytoplasm of hepatoma cells reacting with parasite antigens (not observed in cultures of normal EE parasites at or after 24 h) suggests that EErads somehow continue to break open, shedding all their antigens into the host cell cytoplasm, where they are rapidly degraded. Such a phenomenon is likely to result in all parasite antigens being presented to class 1 MHC. Degradation of foreign antigens

localized in the cytoplasm and presentation of epitopes with class 1 MHC are believed to be rapid processes occurring within 2 to 6 h (27, 44).

Since hepatocytes infected with normal EE parasites are targets for $CD8^+$, CSP-specific T cells (31), access of the CSP to the cytoplasm of the infected cell during the normal development of the EE parasite is strongly implicated (44). It is unclear whether the CSP is unique in this respect by virtue of being shed during invasion (14, 38) or whether like most (if not all [42]) EE antigens, it is confined by the parasitophorous vacuole membrane (1, 2) and some other mechanism(s) operate for delivering it (and perhaps therefore also other parasite proteins or peptides) into the infected host cell cytoplasm.

The antigenic repertoire of the EErads is clearly not limited to the CSP and, based on this limited study, appears to be similar to that of young normal EE parasites. Antigens found early in development in normal EE parasites (Pbl1, Pfhsp70) are also synthesized by many EErads (dose, 10,000 rads), but only a limited number of older EErads synthesize antigens characteristic of maturing EE parasites (W3.5, PMMSA).

The ability of EErads to synthesize antigens appears, however, to be progressively reduced as the radiation dose delivered to the sporozoites is increased (Table 1). Increasing radiation doses delivered to sporozoites also lower the level of protection that can be conferred by them in vivo (28). As the invasion rate of sporozoites seems (at least in vitro) to be unaffected by radiation dose and the CSP is thought not to be synthesized by the EE parasite (50), increased protection might be correlated with increased de novo synthesis of antigens by the EErads, perhaps indicating a role for non-CSP antigens in protection. That CSP might not be the only target for protective $CD8^+$ T cells has also recently been implicated in a closely related rodent malaria species, *Plasmodium yoelii*. In contrast to the situation with *P. berghei* (31, 32), anti-*P. yoelii* CSP $CD8^+$ T cells generated by immunization with a vaccinia virus construct (M. Sedegah et al., Bull. W.H.O., in press) or recombinant salmonellae or proteosomes (W. Weiss et al., Bull. W.H.O., in press) do not confer protection, despite the fact that a polyclonal $CD8^+$ T-cell population generated by irradiated sporozoites does protect animals against sporozoite challenge.

It remains to be established whether antigens among the large repertoire expressed by the liver stage (10, 12, 40, 42, 43; Suhrbier et al., in press) can be targets for protective $CD8^+$ T cells and form the basis of an effective vaccine that does not suffer from the potential problems of polymorphism (9, 25a) and genetic restriction (8, 13) associated with the CSP.

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