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Baculovirus virions displaying *Plasmodium berghei* circumsporozoite protein protect mice against malaria sporozoite infection

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Abstract

The display of foreign proteins on the surface of baculovirus virions has provided a tool for the analysis of protein–protein interactions and for cell-specific targeting in gene transfer applications. To evaluate the baculovirus display system as a vaccine vehicle, we have generated a recombinant baculovirus (AcNPV-CSPsurf) that displays rodent malaria *Plasmodium berghei* circumsporozoite protein (PbCSP) on the virion surface as a fusion protein with the major baculovirus envelope glycoprotein gp64. The PbCSP-gp64 fusion protein was incorporated and oligomerized on the virion surface and led to a 12-fold increase in the binding activity of AcNPV-CSPsurf virions to HepG2 cells. Immunization with adjuvant-free AcNPV-CSPsurf virions induced high levels of antibodies and gamma interferon-secreting cells against PbCSP and protected 60% of mice against sporozoite challenge. These data demonstrate that AcNPV-CSPsurf displays sporozoite-like PbCSP on the virion surface and possesses dual potentials as a malaria vaccine candidate and a liver-directed gene delivery vehicle.

Keywords: Baculovirus; Viral vector; Vaccine; gp64; Malaria; *Plasmodium berghei*; Circumsporozoite protein

Introduction

Recently, the baculovirus *Autographa californica* nuclear polyhedrosis virus (AcNPV) has been shown to be capable of displaying a foreign protein on the virion surface (Grabherr et al., 2001). This display system is achieved by using the major surface glycoprotein of AcNPV, gp64, to display foreign proteins on the virion surface by in-frame fusion between the gp64 signal sequence and mature foreign protein domain. For example, the external domain of HIV envelope protein gp120 was displayed on the virion surface (Boublik et al., 1995). The displayed gp120 was oligomerized on the tip of virions in an active form and possessed binding activity for its ligand, CD4. A baculovirus displaying a functional single-chain antibody fragment specific for the carcinoembryonic antigen targeted to adenocarcinoma cells has also been reported, indicating that baculovirus displaying specific ligand-binding proteins may serve as an effective tool for targeted gene therapy to specific cells (Mottershead et al., 2000).

The baculovirus display system has several potential advantages as a vaccine vehicle. The use of purified virions as immunogens alleviates the need for additional extraneous adjuvants to the vaccine formulation due to the intrinsic immunostimulatory effect often associated with viral immunogens. Additionally, the presentation of an antigen in its proper conformation on the virion surface makes it readily accessible for interactions with cellular components of the immune system. In fact, pathogen antigens such as the HIV envelope protein gp41 (Boublik et al., 1995), rubella virus envelope protein (Mottershead et al., 1997), foot-and-mouth disease virus capsid protein (Tami et al., 2000), and Theileria parva p67 antigen (Kaba et al., 2003) have been ex-

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pressed using this system, and some of them induced high titers of antigen-specific antibodies. To our knowledge, however, the efficacy of the baculovirus display system as a protective vaccine delivery system has not been previously reported.

Baculoviruses have an additional potential for gene delivery to mammalian cells. The host specificity of baculovirus was originally thought to be restricted to arthropods; however, it has been shown that AcNPV can introduce foreign genes into mammalian cell lines, including hepatocytes (Boyce and Bucher, 1996; Hofmann et al., 1995) and other cell types (Shoji et al., 1997). The major prerequisite for generating recombinant baculoviruses for liver- and non-liver-directed gene transfers is the insertion of a mammalian expression cassette into the virus genome (Condreay et al., 1999). Under these conditions, the transduction efficacy reached 100% in human hepatocytes ex vivo (Sandig et al., 1996). The binding of baculovirus to mammalian cells may involve the interaction of gp64 with phospholipids on the cell surface (Tani et al., 2001). Thus, baculovirus possesses two major characteristics relevant to vaccine applications: the display of foreign proteins on the virion surface and the ability to serve as a vehicle for gene transfer to mammalian cells in the absence of cytotoxicity and viral replication.

Malaria kills between 1.5 and 2.7 million people each year, and between 300 and 500 million others fall ill from it, often severely. Clearly, the development of an effective vaccine would greatly facilitate worldwide control of this important parasitic disease. We have focused on developing a novel malaria vaccine based on the circumsporozoite protein (CSP), the major surface antigen of the infective sporozoite stage (Yoshida et al., 2000). Antibodies directed against CSP inhibit the invasion of hepatocytes by sporozoites and CSP-specific $CD8^+$ T cells kill sporozoite-infected hepatocytes (Hoffman et al., 1996). CSP-specific protective immune responses have been induced with various types of subunit vaccines, including synthetic peptides, bacterial and viral vectors, and DNA vaccines (Doolan and Hoffman, 1997; Hoffman et al., 1996). In terms of its biological function, CSP appears to be involved in sporozoite adherence to hepatocytes. Within minutes after entering a blood capillary via a mosquito bite, the sporozoites travel through the bloodstream to the liver where they invade their target cells, the hepatocytes (Sinnis, 1996). This liver-specific invasion is mainly attributed to the region II-plus of CSP, which binds to heparan sulfate proteoglycans (HSPGs) on the hepatocyte membrane (Cerami et al., 1994). Thus, CSP functions both as an immunogen capable of inducing protective immunity and a ligand for liver membrane receptors.

Our strategy for malaria vaccine development is to generate a "sporozoite-like" CSP particle using the baculovirus display system. Immunization with these "sporozoite-like" CSP particles is expected to elicit both neutralizing antibodies and protective cellular immune responses resulting from the effective presentation by antigen-presenting cells. To that end, in this work, we generated a recombinant baculovirus displaying CSP on the virion surface. These recombinant baculovirus virions specifically bound to HepG2 cells through HSPGs. The immunization of mice with the baculovirus virions displaying PbCSP elicited specific antibodies and gamma interferon (IFN-γ) production and conferred protection against *Plasmodium berghei* challenge infection.

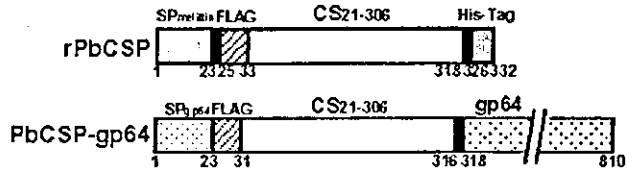

Fig. 1. PbCSP expressed by recombinant baculoviruses shown by schematic representations of rPbCSP and PbCSP-gp64 fusion proteins expressed by AcNPV-CSP and AcNPV-CSPsurf, respectively. Numbers indicate the amino acid positions of each protein. Bold lines indicate junctions. SPmellitin, signal leader peptide of mellitin from honey bees; SP64, signal leader peptide of baculovirus gp64; FLAG, FLAG epitope tag (DYKDDDDK); $CS_{21\text{-}306}$, PbCSP corresponding to amino acids 21 to 306.

Results

Expression of CSP-gp64 fusion protein and display on the virion surface

The *P. berghei* circumsporozoite protein (PbCSP) gene fragment encoding amino acids 21–306, which lacks its signal peptide and hydrophobic C-terminal region, was inserted into the pBACsurf vector to express the PbCSP-gp64 fusion protein on the virion surface. As a control virion without PbCSP at its surface, the same fragment of the PbCSP gene was inserted into the pBACgus1 vector. Recombinant baculoviruses AcNPV-CSP and AcNPV-CSPsurf were generated by cotransfection with pBACgus-CSP and pBACsurf-CSP, respectively. Fig. 1 shows a schematic diagram of the rPbCSP and PbCSP-gp64 fusion proteins. Both proteins contain a FLAG epitope tag at the N-terminus to facilitate their detection.

Lysates of Sf9 cells infected with the recombinant baculoviruses were prepared and lysate proteins were separated by SDS–PAGE followed by immunoblot analysis using the anti-PbCSP monoclonal antibody (MAb) (Fig. 2A) and anti-FLAG MAb (Fig. 2B). Both anti-PbCSP MAb and anti-FLAG MAb recognized rPbCSP expressed by AcNPV-CSP as a doublet with relative molecular masses (M_r) of 48 and 52 kDa (Figs. 2A and B, lanes 2 and 6, respectively), which may be due to posttranslational modification or to degradation. AcNPV-CSPsurf expressed a PbCSP-gp64 fusion protein with an M_r of 120 kDa in infected Sf9 cells (Figs. 2A and B, lanes 3 and 7). As a positive control, the native PbCSP of sporozoites with an M_r of 44 kDa was reacted with anti-PbCSP MAb (Fig. 2A, lane 1).

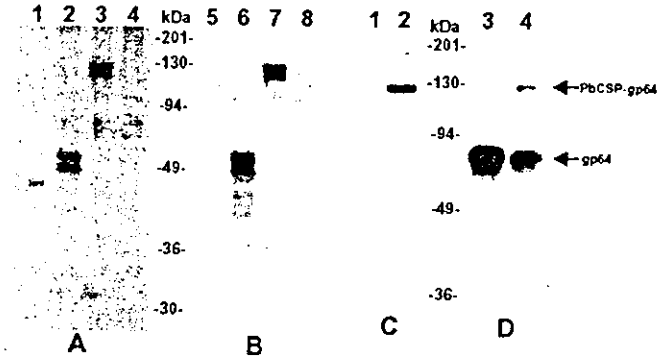

Fig. 2. Western blot analysis of recombinant baculoviruses is shown by (A) immunoblotting of Sf9 cells infected with AcNPV-CSP (lanes 2, 6), AcNPV-CSPsurf (lanes 3 and 7), or wild-type baculovirus (lanes 4 and 8) performed using anti-PbCSP MAb (lanes 1–4) or anti-FLAG M2 MAb (lanes 5–8). As a control, sporozoites isolated from the salivary gland of the *A. stephensi* mosquito were immunoblotted (lanes 1 and 5). (B) Immunoblotting of purified AcNPV-CSP virions (lanes 1 and 3) or purified AcNPV-CSPsurf virions (lanes 2 and 4) was performed using anti-PbCSP MAb (lanes 1 and 2) or anti-gp64 MAb (lanes 3 and 4). Positions of PbCSP-gp64 fusion protein and gp64 are indicated. Baculovirus virions were purified as described under Materials and methods.

To determine whether the PbCSP-gp64 fusion protein is incorporated into the AcNPV virions, budded baculovirus virions were purified from culture supernatants using a 10–60% sucrose gradient and were analyzed by Western blotting using anti-PbCSP MAb (Fig. 2C) or anti-gp64 MAb (Fig. 2D). The 120-kDa fusion protein was detected by anti-PbCSP MAb (Fig. 2C, lane 2). In contrast, no band reactive with anti-PbCSP MAb was seen for purified AcNPV-CSP virions (Fig. 2C, lane 1). Anti-gp64 MAb recognized both the PbCSP-gp64 fusion protein and native gp64 in AcNPV-CSPsurf virions, whereas only native gp64 was detected in AcNPV-CSP virions (Fig. 2D). These data clearly indicate that CSP is incorporated into AcNPV-CSPsurf virions as a CSP-gp64 fusion protein, whereas CSP does not exist in AcNPV-CSP virions.

Direct binding of AcNPV-CSPsurf virions to HepG2 cells

Cerami et al. (1994) demonstrated that CSP on the sporozoite surface serves as the hepatocyte-binding ligand by virtue of its binding to membrane HSPG. To determine whether AcNPV-CSPsurf virions could interact with hepatocytes in a similar manner, we first examined whether PbCSP-gp64 fusion protein incorporated into the virion was capable of binding to HepG2 cells with a specificity similar to sporozoite CSP. Fig. 3 shows that the binding activity of AcNPV-CSPsurf virions to HepG2 is 12-fold higher than those of wild-type AcNPV and AcNPV-CSP virions (non-treatment), indicating that the CSP-gp64 fusion protein is displayed on the surface of AcNPV-CSPsurf virions. Second, since region II-plus at C-terminus of CSP is required for sporozoite binding to hepatocytes, we examined whether the binding of AcNPV-CSPsurf virions to the HepG2 cell was similarly dependent on an interaction with CSP region II-plus. Virion binding was reduced by 50% in the presence of the region II-plus peptide (region II-plus peptide), indicating that interaction of the virion PbCSP-gp64 fusion protein with HepG2 cells specifically involves the CSP region II-plus. Third, we examined whether hepatocyte surface HSPGs act as receptors for the PbCSP-gp64 fusion protein. Preincubation of AcNPV-CSPsurf virions with heparin caused 80% binding inhibition. Furthermore, pretreatment of HepG2 cells with heparinase I resulted in 58% binding inhibition. Since the binding effect was examined at the highest concentration of virus virions prepared, it is not sufficient to show that the binding effect could be saturated. However, the degree of binding inhibition by region II-plus, heparin, and heparinase I is consistent with that observed for CSP mutants of *P. falciparum* (Rathore and McCutchan, 2000). Recently, Rathore et al. (2002) reported that, in addition to region II-plus, CSP has other regions involved in the binding to liver cells, resulting in the partial binding inhibition by region II-plus.

Oligomerization of PbCSP-gp64 fusion protein

It has been reported that the oligomerization of native CSP on the surface of sporozoites may facilitate effective binding to hepatocytes (Cerami et al., 1992, 1994; Sinnis et al., 1994). To examine the degree of oligomerization of the PbCSP-gp64 fusion protein, AcNPV-CSPsurf virions were treated with different concentrations of 2-mercaptoethanol (2-ME) and analyzed by SDS–PAGE. Fig. 4 shows that the

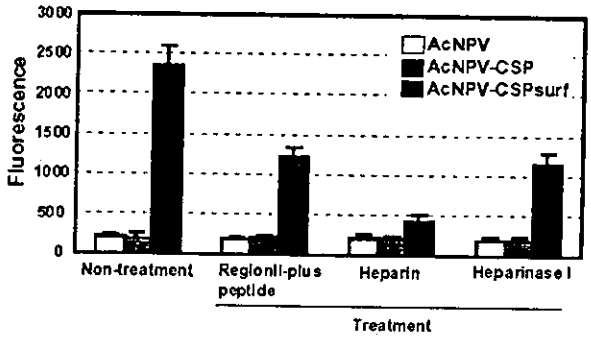

Fig. 3. Binding of AcNPV-CSPsurf virions to HepG2 cells and the effects of region II-plus peptide, heparin, and heparinase I on binding. The following four assays were performed as described under Materials and methods. (1) HepG2 cells were incubated with baculovirus virions (1×10^9PFU) for 1 h followed by the addition of anti-gp64 MAb and alkaline phosphatase-conjugated antimouse IgG. Bound enzyme was measured by a fluorescent substrate. (2) Baculovirus virions (1×10^9PFU) were added to HepG2 cells preincubated with 70 μM region II-plus peptide. Binding activity was measured as described. (3) Baculovirus virions (1×10^9PFU) were incubated with heparin (10 IU/ml) before its addition to HepG2 cells. Binding activity was measured as described. (4) HepG2 cells were preincubated with heparinase I (5 U/ml) for 2 h followed by the addition of baculovirus virions (1×10^9PFU). Binding activity was measured as described. Data are presented as the mean ± SEM of triplicate wells from three independent experiments.

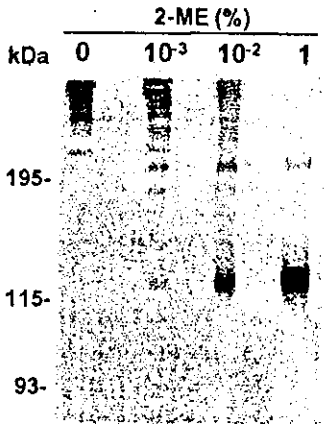

Fig. 4. Western blot analysis of AcNPV-CSPsurf virions under partially reduced conditions is shown. AcNPV-CSPsurf virions were treated with 10^{-3}, 10^{-2}, or 1% 2-ME or without 2-ME before SDS–PAGE. Immunoblotting of these samples was performed using anti-PbCSP MAb, 3D11.

PbCSP-gp64 fusion protein was detected in multiple forms under nonreducing and partially reducing conditions containing 10^{-3}, 10^{-2}, and 1% 2-ME. These results suggest that most of the PbCSP-gp64 fusion protein on the virion surface exists in an oligomeric form.

Protective efficacy of AcNPV-CSsurf against challenge

To study the protective efficacy of AcNPV-CSPsurf virions as a baculovirus-based vaccine in an experimental system emulating the natural route of malarial infection, BALB/c mice immunized intramuscularly (im) with two or three doses of AcNPV-CSPsurf virions were challenged by bites of *P. berghei*-infected mosquitoes. Protection was defined either as the complete absence of blood-stage parasitemia from 3 to 21 days postchallenge (complete protection) or as the delayed onset of parasitemia compared to naïve control groups (partial protection). Although, in our challenge method using mosquito bites, 100% of partially protected mice with delayed onset of parasitemia resulted in death, Sauzet et al. (2001) reported that 1-, 2-, or 3-day delayed onset of parasitemia relative to naïve control mice would represent 80, 96, and 99.2% reductions in liver parasite burden, respectively, indicating that the delayed onset of parasitemia could be utilized as an important parameter of protection efficacy. Therefore, we estimated the protection efficacy by using both complete and partial protection. Parasitemia of naïve control mice occurred 5 days or less after challenge in three experiments. In Experiment 1, 13 of 20 mice (63%) were completely protected. Of the 7 infected mice, 5 achieved partial protection, with 3 mice being delayed by 1 day, 1 mouse being delayed by 2 days, and 1 mouse being delayed by 3 days. Overall, 18 (90%) of 20 mice immunized with AcNPV-CSPsurf virions were partially or completely protected. It is also important to emphasize that inoculation with AcNPV-CSP had no effect on the course of infection relative to naïve controls (Table 1, Experiment 1). Similarly, in Experiments 2 and 3, 90% of mice immunized with AcNPV-CSPsurf virions were partially or completely protected and 60% were completely protected (Table 1, Experiments 2 and 3).

Evaluation of immune responses induced by immunization

To understand the potential relationship between humoral responses and protection in mice immunized with AcNPV-CSPsurf virions im, the prechallenge antibody responses to sporozoites were assessed by an indirect fluorescence antibody test (IFAT). High IFAT titers (mean titer, 1:28,700) were induced in mice immunized im twice or three times with AcNPV-CSPsurf virions in the three experiments (see Table 1). When mice immunized with AcNPV-CSPsurf virions in Experiments 1 and 2 were regrouped into completely protected mice ($n = 19$) or infected mice (partially protected and nonprotected) ($n = 11$), the mean IFAT titer was slightly higher for the protected mice (1:37,600) than for the infected mice (1:13,500) (Fig. 5A).

Table 1
Protection of BALB/c mice after immunization with baculovirus virions displaying PbCSP protein

Immunization group	Route (No. doses)	IFAT titers at challenge	Prepatent period days (range)[a]	No. animals completely and partially protected/ no. challenged (%)[b]	No. animals completely protected/ no. challenged (%)
Experiment 1					
Naive		<1:50	5.0 (5)	0/10 (0)	0/10 (0)
recBAC-CSP	im (3)	<1:50	4.5 (4–5)	0/10 (0)	0/10 (0)
recBAC-CSProof	im (3)	1:2,000–128,000	6.1 (5–8)	18/20 (90)	13/20 (63)
Experiment 2					
Naive		<1:50	4.2 (4–5)	1/10 (20)	1/10 (10)
recBAC-CSProof	im (2)	1:2,000–128,000	6.3 (5–8)	9/10 (90)	6/10 (60)
Experiment 3					
Naive		<1:50	4.3 (4–5)	0/10 (0)	0/10 (0)
recBAC-CSProof	im (3)	1:4,000–128,000	6.2 (5–8)	9/10 (90)	6/10 (60)

[a] The prepatent period in the interval between challenge and the detection of parasitemia
[b] Partial protection was defined as a delayed onset of parasitemia compared with naive control groups (6 or longer prepatent days).

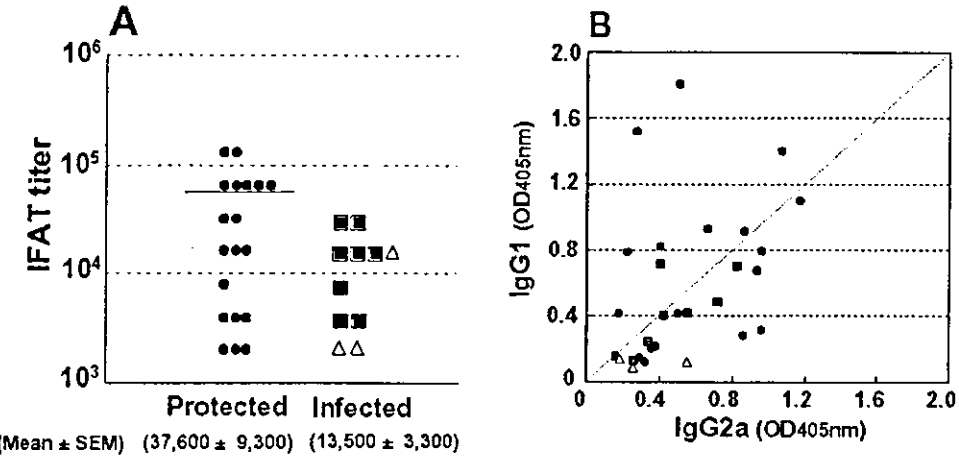

Fig. 5. Humoral responses among protected and infected mice immunized with AcNPV-CSPsurf virions. Sera from immunized mice were collected by tail cut bleeding 2 weeks after the final vaccination followed by challenge. Sera from groups of BALB/c mice immunized with AcNPV-CSPsurf virions in Experiments 1 (n = 20) and 2 (n = 10) (see Table 1) were divided into two groups, protected n = 19 (●) and infected (partial protection n = 8 (□) and nonprotected n = 3 (△) mice. (A) End point IFAT titer to air-dried *P. berghei* sporozoites were plotted in the two groups. Bold bar indicates the IFAT titer at 1:64,000. An IFAT titer at ≥ 1:64,000 led all mice to complete protection. (B) Sera from individual mice were diluted at 1:2000 and tested for IgG1 and IgG2a subclasses by ELISA against rPbCSP. The absorbances of OD_{405} nm for anti-rPbCSP IgG1 and IgG2a were plotted in three groups: complete protection (●), partial protection (□), nonprotection (△).

although this difference was not statistically significant. Seven mice with IFAT titers ≥ 1:64,000 were all protected against the first challenge. In contrast, 11 of 23 mice with IFAT titers ≤ 1:32,000 in both groups were infected, although 2 mice with the lowest IFAT titer (1:2,000) were protected. No detectable CSP-specific antibody was induced in groups of naïve mice or mice immunized with AcNPV-CSP virions, which all became infected. The levels of antibodies of gp64 in groups receiving AcNPV-CSPsurf virions were comparable to those immunized with AcNPV-CSP virions (data not shown). The finding that about half of the mice (12 of 23) with IFAT titers ≤ 1:32,000 in both groups were completely protected may explain variations in the Th-1 and Th-2 ratio bias of the induced immune response. To examine the type of immune response, the levels of IgG1 and IgG2a isotype antibodies specific for recombinant PbCSP (rPbCSP) were measured by ELISA (Fig. 5B). All three nonprotected mice had a low IgG1 antibody (<0.2), whereas 7 mice with a high IgG1 antibody (>0.8), the same mice with IFAT titers ≥1:64,000, were all completely protected. Although the mean ratio of IgG1/IgG2a was significantly higher in the completely protected mice (1.48 ± 0.32) than in the infected mice (0.7 ± 0.13) (Mann–Whitney U test: $P < 0.05$), it is unlikely that complete protection is simply due to Th-2 type response because about 40% of the completely protected mice were plotted in low IgG1 (>0.4) or low IgG1/IgG2a ratios (>1.0) together with the partially protected mice.

Since IFN-γ is a critical component of antimalaria liver-stage protective response, we focused on evaluating the levels of this cytokine secreted in splenocyte culture. Splenocytes were obtained from naïve control mice and mice immunized with AcNPV-CSPsurf virions, and the frequencies of splenocytes secreting IFN-γ in response to PbCSP were measured by ELISPOT. Of four mice immunized with AcNPV-CSPsurf virions, a low but significant level of IFN-γ secretion ($P < 0.05$) was detected in two mice with high IgG1/IgG2a ratios (4.55 and 8.55), whereas no significant IFN-γ secretion was detected in another two mice with low IgG1/IgG2a ratios (0.22 and 0.44).

Protection against rechallenge

To examine the duration of complete protection in these animals, 13 mice completely protected in Experiment 1 (see Table 1) were rechallenged 60 days after the first parasite challenge. All 5 mice (38%) with IgG1 predominant antibodies at the first challenge were protected against rechallenge (Table 2, mice 7–11), whereas another 8 mice with IgG2a predominant antibodies at the first challenge became infected (mice 12–19). The IgG1/IgG2a ratios between the completely protected and the infected mice were significantly different ($P < 0.01$). Noticeably, the IFN-γ responses from the splenocytes of mice protected against rechallenge were dramatically elevated, indicating that priming of the CSP-specific cell-mediated immune response by immunization with AcNPV-CSsurf virions could be boosted by natural sporozoite challenge.

Discussion

In this study, we generated a recombinant baculovirus displaying PbCSP on the virion surface. We chose the baculovirus display system as a new malaria vaccine vehicle because this system has the ability to display a foreign protein in a native form due to the similarities in protein

Table 2
IgG1/IgG2a ratio and the frequencies of IFN-γ-producing cells in AcNPV-CSP surf virions-immunized mice

Groups	IFAT titer	IgG1/IgG2a	IFN-γ ELISPOT (No. SFC/10^4 splenocytes)[c]
Naïve mice			
Mouse 1	<1:50	NT[d]	2.0 ± 0.86
Mouse 2	<1:50	NT	1.0 ± 0.45
Mice immunized im with AcNPV-CSPsurf virions[a]			
Mouse 3	1:2,000	4.55	38.0 ± 5.01*
Mouse 4	1:2,000	0.44	4.67 ± 0.80
Mouse 5	1:16,000	8.55	12.8 ± 2.55*
Mouse 6	1:16,000	0.22	4.67 ± 1.56
Mice protected against rechallenge[b]			
Mouse 7	1:2,000	2.00	437 ± 10.17*
Mouse 8	1:16,000	1.25	135 ± 5.98*
Mouse 9	1:32,000	3.57	256 ± 4.68*
Mouse 10	1:64,000	3.70	387 ± 7.82*
Mouse 11	1:64,000	5.55	NT
Mice infected against rechallenge			
Mouse 12	1:2,000	0.37	NT
Mouse 13	1:4,000	0.97	NT
Mouse 14	1:8,000	0.49	NT
Mouse 15	1:16,000	0.55	NT
Mouse 16	1:32,000	0.56	NT
Mouse 17	1:64,000	0.33	NT
Mouse 18	1:64,000	0.71	NT
Mouse 19	1:128,000	0.82	NT

[a] Mice were immunized im three times with AcNPV-CSPsurf virions. Three weeks after the final immunization, sera and splenocytes were taken from each mouse.

[b] In experiment 1, 13 mice immunized im three times with AcNPV-CSPsurf virions were protected against challenge. Of these mice, 5 mice were protected against rechallenge. Splenocytes were taken from 4 of 5 mice individually 60 days after rechallenge. Sera taken prior to first challenge were used for IFAT and IgG1/IgG2a assays.

[c] Frequencies of splenocytes secreting IFN-γ is response to rPbCSP were measured by ELISPOT. Data represent the mean number ± SEM of SPCs from triplicate cultures of individual mice.

[d] Not tested.

* Statistical significances between naive and immunized mice were determined by Student's t test ($P < 0.05$).

processing and posttranslational modification between insect and mammalian systems. Moreover, compared with other viral-like particles, such as the hepatitis B core particle, baculovirus virions can incorporate not only antigen epitopes but also full-length antigens. AcNPV-CSPsurf virions generated in this study have several notable characteristics, including (1) PbCSP oligomerization on the virion surface and (2) a high binding affinity for HepG2 cells through HSPGs. These properties are in good agreement with those of native CSP on the surface of sporozoites.

The main objective of this study was to test these "sporozoite-like" CSP baculovirus virions for their ability to protect animals against malaria sporozoite challenge. In three independent experiments, 60% of BALB/c mice immunized with AcNPV-CSPsurf virions were completely protected against challenge by mosquito bites. We and other groups have chosen the bites of sporozoite-infected mosquitoes as the route of sporozoite challenge because mosquito-injected sporozoites are more natural and infectious than iv inoculated sporozoites (Leitner et al., 1997; Vaughan et al., 1999; Yoshida et al., 2000). More importantly, it should be noted that sporozoite vaccine trials with humans invariably utilize the bites of sporozoite-infected mosquitoes as their challenge model system (Church et al., 1997; Ockenhouse et al., 1998; Stoute et al., 1997) In this study, the 70–90% of partial and complete protective efficacies against challenge by mosquito bites should be sufficient to pursue the baculovirus display system as a new malaria vaccine.

A limited number of studies have been published addressing the issue of protection against multiple sporozoite challenge infections. In a human trial of the RTS,S malaria vaccine, 88% (7 of 8) of volunteers receiving "RTS,S vaccine 3" were protected against the first challenge by mosquito bites, while 20% (1 of 5) of the protected volunteers were protected against rechallenge (Stoute et al., 1997). In this study, 38% (5 of 13) of mice completely protected against the first challenge, which had PbCSP-specific IgG1 predominant antibodies, were completely protected against rechallenge. Generally speaking, an increased IgG1:IgG2a ratio suggests a bias toward the activation of a Th-2 type cytokine response. In this study, low levels of IFN-γ, a Th-1 type cytokine, were secreted by splenocytes from mice with IgG1 predominant antibody responses following AcNPV-CSPsurf virion immunization. Moreover, sporozoite challenge by bites with infected mosquitoes appeared to boost the IFN-γ response in the protected mice having an IgG1-predominant antibody response. The observation that mice protected against rechallenge were characterized by IgG1-dominant antibodies as well as high levels of IFN-γ is inconsistent with a simple Th-1:Th-2 dichotomous mechanism for immunity to sporozoite infection. Thus, the results of the assays used do not always correlate with the fates of all individual mice after challenge, indicating that AcNPV-CSPsurf virion vaccination could induce a complex interaction between Th-1 and Th-2 responses. This hypothesis requires further investigation in studies measuring the levels of both Th-1 and Th-2 cytokine-producing cells in mice immunized with AcNPV-CSPsurf virions.

Baculovirus AcNPV has been used as a vector for the delivery of genes to mammalian cells in vitro. Based on the finding that AcNPV-CSPsurf has a 12-fold higher binding activity to HepG2 cells than wild-type AcNPV, baculovirus virions displaying PbCSP may be an effective liver-targeted gene delivery vehicle. Baculovirus itself is capable of adherence to mammalian cells using gp64 as a receptor for phospholipids on the host cell surface and viral entry into the cells is mediated by endocytosis following membrane fusion (Tani et al., 2001). In the case of baculovirus virions displaying CSP, hepatocyte-binding activity may be en-

hanced as a result of the interaction of the virion with both gp64 and CSP receptors on the hepatocyte surface. The baculovirus gene transfer system offers considerable potential for development as a gene delivery vehicle due to its lack of pathogenicity in mammalian cells, its long history of safe usage in research laboratories, its large capacity to harbor foreign DNA, and the production of high virus titers in tissue culture. However, the in vivo applicability of baculovirus-mediated gene transfer may be hampered by its susceptibility to the complement system. Recently, a baculovirus displaying a decay-accelerating factor (DAF) on the virion surface has been shown to exhibit strong complement resistance in in vitro assays (Huser et al., 2001). The use of a baculovirus displaying both DAF and CSP would be expected to improve its effectiveness for liver-directed gene transfer in vivo.

Another particularly important consideration for vaccine use in developing countries is its cost effectiveness and the reuse of multiple foreign antigens over time. Baculovirus displaying an epitope tag linked to target antigens, such as the FLAG epitope used in this study, could easily be purified from a serum-free insect culture supernatant using a one-step affinity column. The simplicity of production and purification should translate to the economical large-scale manufacturing of this vaccine. Antivector immunity may seriously reduce the reuse potential of live viral or bacterial vectors by the inhibition of vector proliferation. A passively derived maternal antibody may also be a potential hurdle for vaccination in children less than 1 year of age. Unlike such live vectors, baculovirus virions displaying foreign antigens are subunit vaccines and could induce immunity against foreign antigens in the presence of antibaculovirus immunity. In addition, baculovirus virions incapable of replicating DNA in mammalian hosts may provide the greatest level of safety.

In summary, we report for the first time that a baculovirus display system may be effectively used as a vaccine vehicle in the absence of extraneous immunological adjuvants to provide protective immunity against infection. The exceptional immunogenicity of this vaccine vehicle may be due to the optimal display of native epitopes in oligomeric arrays as well as the potential carrier and adjuvant effects of viral structural proteins. The ultimate goal of any vaccination strategy is to induce a specific, long-lasting immune response that protects the individual from infection with a given pathogen.

Future studies will attempt to improve the efficacy of this baculovirus display vaccine system using prime-boost immunization strategies or the addition of a blood-stage antigen, such as the merozoite surface protein 1, to the display system. The value of this system for in vivo liver-directed gene transfer and the extension of these studies to evaluate the expression of transferred genes within hepatocytes also merit further investigation.

Materials and methods

Mice, parasites, and mosquitoes

Female BALB/c mice were obtained from SEASCO (Saitama, Japan) and used at 7 to 8 weeks of age in all experiments. The *P. berghei* ANKA strain was maintained by cyclical passage through BALB/c mice and *Anopheles stephensi* (SDA 500 strain). *P. berghei*-infected mosquitoes taken 14 days after an infectious blood meal were used to challenge immunized and naïve control mice.

Plasmid construction

A baculovirus transfer vector, pBACsurf-CSP, was constructed by excision of the gene fragment encoding for amino acids 21–306 of PbCSP from pcDNA-CS87 (Yoshida et al., 2000) by digestion with *Pst*I and *Sma*I. The excised fragment was cloned into the *Pst*I/*Sma*I sites of pBACsurf (Novagen, Madison, WI). A baculovirus transfer vector, pBACgus-CSP, was constructed by excision of the gene fragment encoding amino acids 21–306 of PbCSP from pcDNA-CS87 by digestion with *Bam*HI and *Not*I and ligation into the *Bam*HI/*Not*I sites of pBACgus-1 (Novagen).

Recombinant baculoviruses and cells

The recombinant baculoviruses AcNPV-CSPsurf and AcNPV-CSP were generated in *Spodoptera frugiperda* (Sf9) cells by cotransfection of the recombinant transfer plasmids pBAC-CSPsurf and pBAC-CSP, respectively, with BacVector-3000 DNA (Novagen) according to the manufacturer's protocol. Baculoviruses were grown and assayed in Sf9 cells using SF900-II medium (Life Technologies, Inc., Rockville, MD) supplemented with 1% penicillin and 100 U/ml of streptomycin at 27°C. For purification of the recombinant baculoviruses, culture supernatants were harvested 4 days after infection, and budded virions were purified on 10–60% (w/v) sucrose gradients as described previously (Sandig et al., 1996). The virions were resuspended in phosphate-buffered saline (PBS) and infectious titers were determined by plaque assay. For protein production, High Five cells derived from a *Trichoplusia ni* egg (Invitrogen, San Diego, CA) were maintained in Ex-Cell 405 medium (JRH Bioscience, Lenexa, KS) as described previously (Yoshida et al., 1999). Hepatoma cell line HepG2 cells (American Type Cell Culture, Rockville, MD) were maintained at 37°C in RPMI 1640 supplemented with 10% fetal calf serum (FCS), 1% penicillin, and 100 U/ml of streptomycin.

Expression and purification of recombinant PbCSP

High Five cells (1.5×10^6 cells/ml) were infected with AcNPV-CSP at a m.o.i of 3 and incubated at 27°C for 3 days. Recombinant PbCSP (rPbCSP) was purified from

culture supernatants by affinity chromatography on a Ni-NTA Superflow column (Qiagen GmbH, Hiden, Germany) following chromatography on a Q-Sepharose anion exchange column (HiPrep 16/10 QXL, Pharmacia Biotech, Uppsala, Sweden). The purity of the protein was checked by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), and the protein yield was estimated by comparison with BSA standards. The highly purified rPbCSP was used as an enzyme-linked immunosorbent assay (ELISA) antigen.

Immunoblotting

Protein extracts from baculovirus-infected insect cells and baculovirus virions were prepared as described previously (Mottershead et al., 2000). The protein samples were separated on a 6 or 8% SDS–PAGE gel, transferred to an Immobilon Transfer Membrane (Millipore, Bedford, MA), and probed with either the anti-PbCSP monoclonal antibody (MAb) 3D11 (kindly provided by Dr. Ruth Nussenzweig), anti-FLAG M2 MAb (Eastman Kodak Co., Rochester, NY), or anti-AcNPV gp64 MAb (BD Biosciences Clonetech, Palo Alto, CA) as indicated. Bound antibodies were subsequently detected as described previously (Yoshida et al., 1999).

Cell-binding assay

Assays were performed according to the methods described by Rathore and McCutchan (2000). In brief, HepG2 cells at a density of 1×10^5 cells per well were fixed with 4% paraformaldehyde followed by blocking with TBS (20 mM Tris–HCl, pH 7.5, 150 mM NaCl) containing 1% bovine serum albumin (BSA) in a 96-well black plate (Corning Costar, Tokyo, Japan). Baculovirus virions (1×10^9 PFU) were incubated with cells for 1 h, and cells were washed to remove unbound virions and incubated with anti-gp64 MAb for 1 h followed by alkaline phosphatase-coupled antimouse IgG for 30 min. The 4-methylumbelliferyl phosphate (4-MUP) liquid substrate system (Sigma-Aldrich, Tokyo, Japan) was used as substrate, and fluorescence was measured using Fluoroskan Ascent (Thermo Labsystems, Vantaa, Finland) with excitation at 350 nm and emission at 460 nm according to the manufacturer's instructions. All of the experiments were repeated three times.

Binding inhibition by synthetic peptide, heparin, and heparinase I

Region II-plus synthetic peptide EWSQCNVTCGSGIRVRKRKGSN (Eichinger et al., 1986) was purchased from Hokkaido System Science (Sapporo, Japan). For competition assays using the region II-plus synthetic peptide, HepG2 cells were incubated with 70 μM region II-plus synthetic peptide for 2 h before the addition of baculovirus virions (1×10^9 PFU). The cell-binding assay was performed by incubation with anti-gp64 MAb as described above using the 4-MUP detection system.

To evaluate the effect of heparin on the binding activity of baculovirus virions, baculovirus virions (1×10^9 PFU) were preincubated with 10 IU/ml of heparin (Sigma-Aldrich) for 15 min at 37°C before addition to the HepG2 cells. The cell-binding assay was performed using the 4-MUP system followed by incubation with anti-gp64 MAb as described above.

To determine the effect of HSPGs on the binding activity of baculovirus virions, HepG2 cells were treated with 5 U/ml of heparinase I (Sigma-Aldrich) in 10 mM phosphate buffer (pH 6.0) containing 150 mM NaCl, 3 mM KCl, 0.5 mM $MgCl_2$, 1 mM $CaCl_2$, 0.1% glucose, and 0.5% BSA. Cells were washed once in PBS and then incubated with baculovirus virions (1×10^9 PFU). The cell-binding assay was performed by incubation with anti-gp64 MAb as described above. All experiments were repeated three times.

Immunization and challenge infections

Experiment 1

Mice were immunized im three times at 4-week intervals with 1×10^8 PFU of baculovirus virions. Two weeks after the third immunization, all groups of mice were anesthetized and individually exposed to the bites of at least five sporozoite-infected *A. stephensi* mosquitoes over 5 min. After feeding, the mosquitos' salivary glands were dissected and it was microscopically confirmed that 100% of the mosquitoes used for blood feeding were sporozoite-infected, as described previously (Leitner et al., 1997; Yoshida et al., 2000). Beginning 3 days after challenge, mice were checked daily for *P. berghei* infection by the microscopic examination of Giemsa-stained thin smears of tail blood. A minimum of 25 fields (× 400) was read before a mouse was determined to be negative for infection. Mice with negative blood smears were monitored for blood parasites for up to 21 days after challenge. Sixty days after challenge, protected mice were rechallenged by the bites of *P. berghei*-infected *A. stephensi* as described above.

Experiment 2

Mice were immunized twice at 3-week intervals via the im route with 1×10^8 PFU of baculovirus virions. Two weeks after the second immunization, all groups of mice were challenged by bites of *P. berghei*-infected *A. stephensi* as described above.

Experiment 3

Mice were immunized three times at 3-week intervals via the im route with 1×10^8 PFU of baculovirus virions. Two weeks after the third immunization, all groups of mice were challenged by bites of *P. berghei*-infected *A. stephensi* as described above.

Antibody assays

Sera from immunized mice were collected by tail bleeds 2 weeks after the final vaccination, prior to sporozoite challenge. *P. berghei* sporozoite-specific IFAT titers were determined using salivary gland sporozoites produced from *P. berghei*-infected *A. stephensi* mosquitoes as described previously (Charoenvit et al., 1987). In brief, twofold serial dilutions of sera from individual mice were reacted on multispot antigen slides containing 1000 air-dried sporozoites, and antibodies were detected using fluorescein isothiocyanate-conjugated rabbit antimouse IgG.

For analysis of IgG subclass antibodies against PbCSP, a 96-well assay plate (Corning Costar) was coated with rPbCSP at 1 mg/ml in carbonate buffer (pH 9.6) and then blocked with 1% BSA (Sigma-Aldrich) in TBS buffer. Sera from individual mice were diluted 1:2000 in TBS and added to the appropriate wells in triplicate, followed by peroxidase-conjugated rat antimouse IgG1 or IgG2a antibodies (Zymed, San Francisco, CA). Peroxidase substrate solution [H_2O_2 and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonate)] was added to each well and the absorbance value at 405 nm was measured using a Multiskan BICHROMATIC microplate reader (Thermo Labsystems, Vantaa, Finland). Data were analyzed and compared by the Mann–Whitney *U* test.

ELISPOT assays

PbCSP-specific IFN-γ-producing cells were determined by enzyme-linked immunospot (ELISPOT) as described previously (Currier et al., 2002). In brief, 96-well plates (MultiScreen IP Filtration plate MAIPS45 10, Millipore) were coated with 15 μg/ml of the antimouse IFN-γ MAb R4-6A2 (PharMingen, San Diego, CA) in 50 μl of 0.1 M $NaHCO_3$. After overnight incubation at 4°C, the wells were washed with PBS and blocked for 2 h at 37°C with 200 μl of RPMI 1640 containing 10% FCS. Splenocytes from immunized mice were resuspended to 1×10^7 cells/ml (1×10^6 cells/well) and placed in triplicate into antibody-coated wells, and rPbCSP (10 μg/ml) was added to each test well. After 20 h of incubation at 37°C, the plates were washed six times with PBS containing 0.05% Tween 20 (PBST) and once with water, incubated for 2 h at room temperature with a solution of 1 mg/ml of biotinylated antimouse IFN-γ MAb XMG1.2 (PharMingen, San Diego, CA), washed six times with PBST, and incubated for 2 h with 50 μl of a 1 μg/ml solution of streptavidin–alkaline phosphatase conjugate (PharMingen), both at room temperature. Spots were developed by adding 100 μl of 5-bromo-4-chloro-3-indolylphosphate *p*-toludin salt/nitroblue tetrazolium chloride (BCIP/NBT Alkaline Phosphatase Substrate kit IV; Vector Laboratories, Inc., Burlingame, CA), and reactions were stopped by a water wash. Spot-forming cells (SFCs) were enumerated using the KS ELISPOT system (Carl Zeiss, Hallbergmoos, Germany). Data represent the means ± SEM of SFCs from triplicate cultures of individual mice. Data were analyzed and compared by Student's *t* test.

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Prospective Clinical Evaluation of the Serologic Tuberculous Glycolipid Test in Combination with the Nucleic Acid Amplification Test

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We have conducted a prospective controlled multicenter study to evaluate differences in the levels of clinical utility of the tuberculous glycolipid (TBGL) serodiagnostic test and the nucleic acid amplification test in patients with smear-negative active pulmonary tuberculosis (TB). The TBGL test and the PCR test were individually not so useful for the rapid diagnosis of smear-negative active pulmonary TB. However, clinical utility was considerably improved by using the TBGL test and the PCR test in combination, especially in patients with smear-negative and culture-negative active pulmonary TB and in patients with minimally advanced lesions.

For early detection and treatment of tuberculosis (TB) patients before transmission of TB bacilli, it is important to improve diagnostic techniques. Recently, nucleic acid amplification (NAA) tests have been successfully established as methods for rapid and accurate diagnosis of active pulmonary TB (6, 13, 14). For patients with smear-negative pulmonary TB, however, NAA diagnostic sensitivity was reported to be approximately 50%. Sensitivity was found to be extremely low (5 to 20%) in smear- and culture-negative cases (2, 3, 4, 9, 10). A new serological tuberculous glycolipid (TBGL) test has been developed, and this test has been reported to be useful for the rapid diagnosis of active pulmonary TB (8, 11). We considered rapid diagnosis using the TBGL test to be effective for patients with smear-negative active pulmonary TB, especially when combined with the NAA test. For this report, we have conducted a prospective controlled multicenter study to evaluate the clinical usefulness of the NAA and TBGL tests.

Patients were referred as potential participants for the study between April 1999 and March 2000. They were diagnosed (according to clinical symptoms and signs and chest X-ray findings during the first visit in each respiratory institute) as having smear-negative active TB. We obtained informed consent before participation and prospectively studied all patients. Patients with a medical history of smear tests positive for acid-fast bacilli were excluded from this study. Patients who were defined to be smear positive at the admission examination and patients with nontuberculous mycobacteriosis, cured pulmonary TB, or pleurisy were excluded. All patients requiring anti-TB chemotherapy were admitted to the hospitals. Patients with other respiratory diseases (such as lung cancer, pulmonary bacterial infections other than mycobacterial, or interstitial pneumonia) who were undergoing differential diagnosis for active pulmonary TB were enrolled as controls during same period. Additional evaluation procedures, such as bronchoscopy, were performed at the discretion of the treating physician. Hospital records were reviewed to abstract demographic data and clinical information. Eligibility and clinical records of each enrolled patient were reviewed at an extramural meeting. No patients identified as having human immunodeficiency virus infection were included in this study.

Sputum specimens for both smear staining and culturing were obtained on three consecutive days after admission. The sputum specimens were digested and decontaminated with a solution of 2% sodium hydroxide (NaOH) and *N*-acetyl-L-cysteine (NALC) and then centrifuged for 15 min at 3,000 × *g*. The supernatant was removed, and the remaining sediment was mixed in a 1:10 dilution with sterile water. The processed sample was stained with Auramine O fluorochrome and examined by fluorescent microscopy. Ziehl-Neelsen acid-fast staining was used to confirm the presence of acid-fast bacilli. Mycobacterial cultures were grown by inoculating 0.1 ml of the processed sample into tubes of Ogawa medium (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) (12) and/or into the liquid medium of a Mycobacteria Growth Indicator Tube system (Beckton Dickinson) according to the manufacturers' instructions. Identification to species level of *Mycobacterium tuberculosis* and other nontuberculous mycobacteria (NTM) was determined by a DNA-DNA hybridization method with a DDH Mycobacteria apparatus (Kyokuto Pharmaceuticals, Tokyo, Japan).

The Roche Amplicor Mycobacterium test (Amplicor MTB) was performed according to the manufacturer's instructions (Roche Diagnostics, Laval, Quebec, Canada). Sputum specimens for NAA were collected on admission. Aliquots of 0.1 ml were prepared from all sputum specimens for NAA. The TBGL serodiagnostic test (Kyowa Medex Co., Ltd., Tokyo, Japan) was performed as described previously (11). Serum specimens were obtained 1 day before chemotherapy and stored

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TABLE 1. Subject profiles and TBGL antibody titers

Patient group[a]	No. of patients (male/female)	Age (mean ± SD)	Anti-TBGL titer (U/ml) (mean ± SD)
Smear-negative active pulmonary TB	111 (78/33)	61.0 ± 20.7	6.2 ± 8.4
Culture positive	70 (51/19)	63.2 ± 20.3	7.0 ± 9.0
Culture negative	41 (27/14)	57.3 ± 20.8	5.0 ± 7.3
Other respiratory disease	52 (52/16)	66.9 ± 18.4	1.2 ± 1.9[b]
Lung cancer	21 (17/4)	70.3 ± 13.6	1.0 ± 1.2[b]
Pneumonia	16 (8/8)	62.9 ± 26.3	1.5 ± 1.8[b]
Lung abscess	5 (3/2)	58.0 ± 10.7	2.4 ± 4.7
Others	10 (8/2)	70.7 ± 13.9	0.7 ± 0.7[b]

[a] Among the patients who were referred as potential participants for the study, those who were defined to be smear positive for active pulmonary TB (94 patients) and those with nontuberculous mycobacteriosis (61 patients), pleuritis (8 patients), or old TB (10 patients) were excluded.
[b] $P < 0.05$ versus smear-negative pulmonary TB.

at −20°C until assay of TBGL antibody titers. All technicians carried out the NAA and the enzyme-linked immunosorbent assay such that they were blind to the final diagnosis.

Active TB was confirmed when cultures of respiratory secretions (sputum or bronchial lavage) were positive for *M. tuberculosis*. Active clinical TB (smear negative and culture negative) was confirmed when there was a compatible abnormal chest X ray which showed improvement, as judged by independent review of the chest X ray, after treatment for 2 to 3 months with three or four antituberculosis drugs. Agreement of three specialists in their separate respiratory institutions was required to confirm the clinical diagnosis. The extent of TB lesions was classified according to the guideline of the Committee on Revision of Diagnostic Standards (5). A meeting of an investigative commission was held during which the investigators confirmed the criteria for diagnosis, especially by X ray. According to the American Thoracic Society TB classification system (1, 7), the active clinical TB group was class 3. NTM was diagnosed, in accord with American Thoracic Society criteria (15), by isolation of potentially pathogenic NTM from the respiratory secretions (sputum or bronchial lavage). Diagnoses of other respiratory diseases (cancer, lung infections, other bronchial diseases, etc.) were based on chest X rays compatible with the original diagnosis, diagnoses by the treating physicians, and all other clinical information.

Statistical analyses were performed with a computer-based statistical package (Statcel; OMS, Ltd., Saitama, Japan). The values are expressed as means ± standard deviations (SD) and were determined by a Student *t* test or a multiple comparison test (Scheffe's F test) for a single factor. Differences in sensitivity between diagnostic methods were evaluated by a Fisher's exact probability test.

As shown in Table 1, 111 patients were diagnosed with smear-negative active pulmonary TB. There were 70 patients who were culture positive for *M. tuberculosis* and 41 who were culture negative. The samples from the smear-negative active pulmonary TB group gave significantly ($P < 0.05$) higher TBGL antibody titers than those in the other respiratory disease group (Table 1 and Fig. 1), leading to a high level of specificity (88.5%) as reported previously (11).

The sensitivity of the TBGL test was compared with that of the NAA test for the smear-negative active pulmonary TB group (Fig. 2). For this group, the sensitivity of the TBGL test (56.8%) was comparable to that of the NAA test (52.3%). In the subgroup of culture-positive cases, the sensitivity of the NAA test was higher (72.9%) than that of the TBGL test

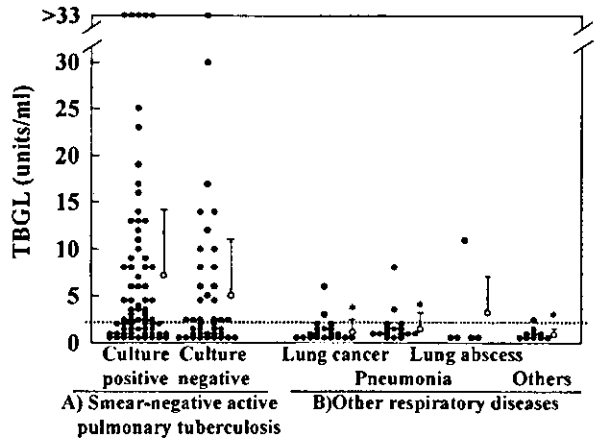

FIG. 1. Distribution of TBGL antibody titers in patient groups. The dotted line indicates a cutoff point (2 U/ml). Closed circles indicate individual values. Open circles indicate means plus SD. Asterisks indicate values significantly different from those for patients with smear-negative active TB ($P < 0.05$).

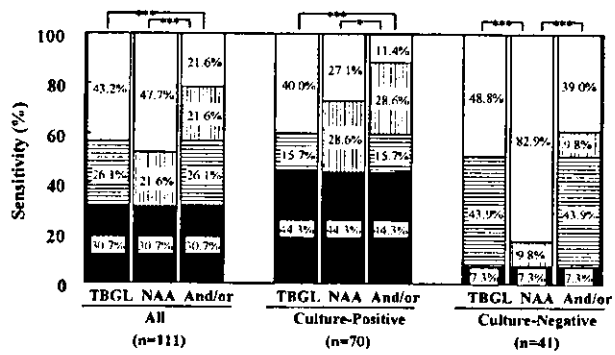

FIG. 2. Sensitivity of TBGL test and NAA test, either alone or in combination, for patients with smear-negative active pulmonary TB. Symbols: open, not detected; vertical stripe, detected by NAA alone; horizontal stripe, detected by the TBGL test alone; closed, detected by both the TBGL test and NAA. Significant differences are indicated (*, $P < 0.05$; ***, $P < 0.001$).

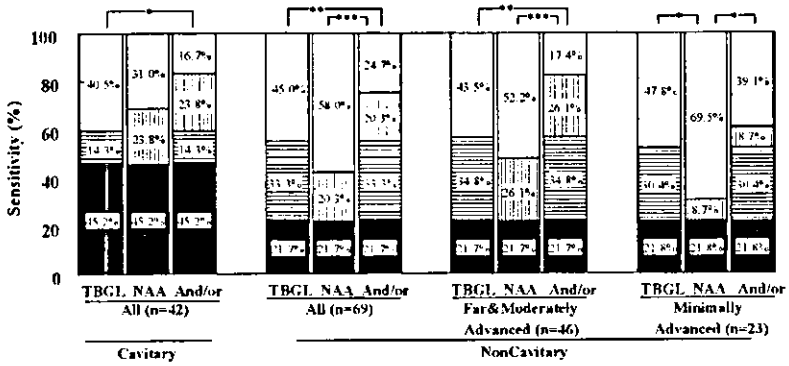

FIG. 3. Sensitivity of TBGL test and NAA test, either alone or in combination, according to chest X-ray findings for patients with smear-negative active pulmonary TB. The extent of TB lesions was classified according to the guideline of the Committee on Revision of Diagnostic Standards (5). Symbols: open, not detected; vertical stripe, detected by NAA alone; horizontal stripe, detected by the TBGL test alone; closed, detected by both the TBGL test and NAA. Significant differences are indicated (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

(60.0%). In the subgroup of culture-negative cases, however, the sensitivity of the NAA test was significantly lower (17.1%) than that of the TBGL test (51.2%). Using the TBGL test and the NAA test combined, the sensitivity of diagnosis for smear-negative active pulmonary TB was significantly increased to 78.4%. In patients with smear-negative and culture-positive active pulmonary TB, the sensitivity of the NAA test was 72.9%, but when used in combination with the TBGL test, the sensitivity was significantly increased and diagnosed 88.6% of patients as having active pulmonary TB. For patients with smear- and culture-negative pulmonary TB, using the combined TBGL and NAA tests significantly improved sensitivity (61%). In fact, 30 of 53 (56.6%) smear-negative and NAA-negative cases and 18 of 34 (53.0%) smear-negative, culture-negative, and NAA-negative cases were TBGL positive.

The sensitivities of the TBGL test and the NAA test were compared on the basis of the presence or absence of cavitary lesions in chest radiographs of patients, as shown in Fig. 3. In 42 cases of patients with cavitary lesions, the sensitivity of the NAA test was slightly higher (69.0%) than that of the TBGL test (59.5%). In 69 cases of noncavitary lesions, an assessment was performed according to the spread of lesions, demonstrating that the sensitivity of the TBGL test was higher than that of the NAA test, especially in cases with minimally advanced lesions.

The use of the TBGL test in combination with the NAA test significantly improved the sensitivity of diagnosis of pulmonary TB from 42.0 to 75.3%. In the subgroup of cases with minimally advanced active pulmonary TB without cavitary lesions, the use of both tests in combination significantly improved sensitivity from 30.5 to 60.9%. Also, in terms of chest radiographic findings, the TBGL test was more sensitive than the NAA test for the cases with noncavitary lesions, irrespective of the spread of lesions. These results indicated that the serodiagnostic test can improve the differential diagnosis of smear-negative TB, especially at early stages before tuberculous bacilli can be detected in the sputum.

The TBGL test and the PCR test were not so useful individually for the rapid diagnosis of smear-negative active pulmonary TB. However, clinical utility was considerably improved by using the TBGL test and the PCR test in combination, especially in patients with smear-negative and culture-negative active pulmonary TB and in patients with minimally advanced lesions.

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THE JOURNAL OF IMMUNOLOGY

CUTTING EDGE

Cutting Edge: Major CD8 T Cell Response to Live Bacillus Calmette-Guérin Is Mediated by CD1 Molecules[1]

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MHC class I-restricted CD8$^+$ T cells are a crucial component of the host defense against mycobacterial infection in mice, but it has often proved very difficult to identify the CD8 T cell response in humans. Human group 1 CD1 molecules (CD1a, -b, -c) mediate MHC-independent presentation of mycobacteria-derived lipid and glycolipid Ags to CD8$^+$ T cells, and their intracellular localization to the endocytic system may favor efficient monitoring of phagosome-resident mycobacteria. Here, we show that bacillus Calmette-Guérin (BCG)-immunized subjects contain a significant circulating pool of CD8$^+$ T cells that recognize BCG-infected DCs in a CD1-dependent, but MHC-independent, manner. These CD1-restricted T cells efficiently detected live, rather than dead, BCG and produced IFN-γ, an important cytokine for protection against mycobacterial infection. These results emphasize that lipid-reactive CD8$^+$ T cells may contribute to host defense against mycobacterial infection. The Journal of Immunology, 2003, 170: 5345–5348.

Besides the prominent role of CD4$^+$ T cells and IFN-γ that they produce in host defense against mycobacterial infection, CD8$^+$ T cells appear to mediate distinct but complementary pathways for controlling intracellular mycobacteria (1, 2). Evidence obtained from analysis of TAP- and CD1d-deficient mice as well as an earlier study of mice lacking the expression of β_2-microglobulin unequivocally demonstrated a significant contribution of MHC class I-restricted CD8$^+$ CTLs in clearing mycobacterial infection in mice (3, 4). In humans, the potential role of CD8$^+$ T cells in protection against mycobacterial infection has been appreciated partially by isolating and characterizing mycobacteria-specific, MHC class I-restricted CD8$^+$ T cell lines (5). These studies focused on macrophages, the well-known reservoir for mycobacteria, as CTL target cells, that were either infected with mycobacteria or pulsed with mycobacteria-derived peptide Ags.

Recently, the universe of human CD8$^+$ CTLs has been expanded to include those that recognize mycobacterial cell wall-derived lipid and glycolipid Ags in the context of group 1 CD1 molecules (CD1a, -b, and -c) expressed prominently and almost exclusively on dendritic cells (DCs)[3] (6). Given that DCs are the most efficient APCs in the immune system and that, besides macrophages, DCs represent another important reservoir for mycobacteria (7), group 1 CD1-dependent activation of CD8$^+$ T cells may occur during the course of mycobacterial infection (8). In addition, CD1 molecules are expressed intracellularly in endocytic subcompartments where lipid Ags derived from phagocytosed mycobacteria are known to traffic (9, 10), raising the possibility that CD1 molecules may be situated to efficiently monitor infection with live mycobacteria. Indeed, group 1 CD1-restricted CD8$^+$ T cell lines were isolated from healthy subjects as well as patients with mycobacterial infection, and their outstanding ability to recognize mycobacteria-infected cells and kill the intracellular organisms has been noted (11). Thus, despite the previous observations that a majority of human circulating CD8$^+$ T cells may recognize mycobacteria-infected cells in a CD1-independent manner (12, 13), we reasoned that a circulating pool of CD1-restricted CD8$^+$ T cells reactive to live mycobacteria might be detected in those who have established augmented cell-mediated immunity against mycobacterial infection. In the present study, we show that a sizable pool of such CD8$^+$ T cells exists in the peripheral blood of individuals inoculated with live *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) and that these CD1-restricted T cells produce IFN-γ in response to live BCG-infected DCs. Lipid Ag-reactive CD8$^+$ T cells may therefore contribute to host defense against mycobacterial infection.

Materials and Methods

BCG culture and infection

The Tokyo 172 strain of BCG was grown in 7H9 medium and the extraction with chloroform/methanol was performed as described previously (14). TLC of the organic extract was conducted on 200-μm silica-coated glass TLC plates developed in 50% sulfuric acid as described (14). For infection experiments, BCG was harvested at its midlog phase growth, washed, and suspended in

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[3] Abbreviations used in this paper: DC, dendritic cell; BCG, bacillus Calmette-Guérin; GMM, glucose monomycolate; PPD, purified protein derivative.

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RPMI 1640 (Life Technologies, Gaithersburg, MD) supplemented with 10% FCS (HyClone Laboratories, Logan, UT). Without prior sonication, the suspension was passed through a 5-μm pore size filter to obtain single-cell bacteria. The viability of bacteria was constantly >90%. The BCG preparation was divided into equal aliquots; one aliquot was incubated for 30 min at 85°C to kill the bacteria, whereas the other aliquot was left at room temperature. Human peripheral blood monocytes and monocyte-derived DCs were isolated as described (15) and incubated for 4 h with the BCG preparations at the multiplicity of infection of 10. The infected cells were then washed and incubated for an additional 14 h in RPMI 1640 complete medium without antibiotics. At the end of the culture, the cells were harvested, irradiated, and used as APCs in T cell stimulation assays and ELISPOT assays.

T cell transfectants stimulation assays

Reconstitution of the LDN5 TCR in TCR-deficient Jurkat cells (J.RT3/LDN5) was conducted as described (14). The J.RT3/LDN5 cells (5×10^4/well) were cultured for 20 h with either BCG-infected, glucose monomycolate (GMM)-pulsed, or untreated DCs (5×10^4/well), and the amount of IL-2 released into the supernatants was measured as described (14).

ELISPOT assays

PBLs were obtained from 11 healthy Japanese subjects (donors 1–11) who had positive purified protein derivative (PPD) test conversion due to BCG vaccine inoculation at their infancy. The subjects included 8 men (donor 1, 30 years old; donor 3, 32 years old; donor 5, 30 years old; donor 6, 32 years old; donor 7, 34 years old; donor 8, 33 years old; donor 9, 30 years old; donor 10, 44 years old) and 3 women (donor 2, 32 years old; donor 4, 33 years old; donor 11, 33 years old). After incubation of the PBLs at 4°C in the presence of anti-CD19 and anti-CD56 Abs (both from BD PharMingen, San Diego, CA) together with Abs against either CD4 (OKT4) or CD8α (OKT8) (15), the labeled cell populations were removed by two cycles of separation with magnetic beads coated with goat anti-mouse IgG Abs. The efficiency in depletion of the corresponding T cell populations was determined by flow cytometry as described (14). The CD8 T cell-enriched and the CD4 T cell-enriched populations (1×10^5/well) were separately incubated for 24 h with APCs (1×10^4/well), and ELISPOT assays were performed as described (5). In some experiments, the culture was performed in the presence of saturating amounts of either W6/32 (anti-MHC class I) (16, 17), L243 (anti-HLA-DR) (18), 10H3.9.3 (anti-CD1a) (14), BCD1b3.1 (anti-CD1b) (14), F10/21A3.1 (anti-CD1c) (14), or RPC5.4 (control; obtained from the American Tissue Culture Collection, Manassas, VA). Each experiment was repeated two to four times to confirm reproducibility of the results.

Results

A CD1b-restricted T cell line recognized DCs infected with live BCG

To evaluate the ability of CD1 to monitor mycobacterial infection, we first used a CD1-restricted T cell line, LDN5 (19), that was specific for GMM produced by several species of mycobacteria. Indeed, BCG grown for 7 days in 7H9 medium supplemented with 3% D-glucose produced a significant amount of GMM in culture (Fig. 1*A*, arrowhead). To examine GMM recognition in BCG-infected $CD1^+$ cells, monocyte-derived DCs were infected with either live or heat-killed BCG and used as APCs. For detection of GMM presentation by these DCs, TCR-deficient Jurkat cells (J.RT3) reconstituted by transfection with the LDN5 TCR α- and β-chains (J.RT3/LDN5) (14) were used as responder cells that were capable of recognizing GMM in the context of CD1b molecules. As expected, GMM-pulsed DCs, but not unpulsed cells, were able to stimulate the J.RT3/LDN5 cells to produce IL-2 (Fig. 1*B*, DC + GMM and untreated DC, respectively). DCs infected with live BCG were efficiently recognized by J.RT3/LDN5 cells, indicating that GMM was presented by live BCG-infected DCs (Fig. 1*B*, DC + live BCG). Despite the fact that phagocytosis of live and dead BCG occurred in similar efficiency and the bacilli contained a significant amount of GMM in their cell wall (Fig. 1*A*), DCs treated with dead BCG were incapable of or markedly inefficient in stimulating J.RT3/LDN5 cells. The level of IL-2 produced by J.RT3/LDN5 in response to dead BCG-infected DCs

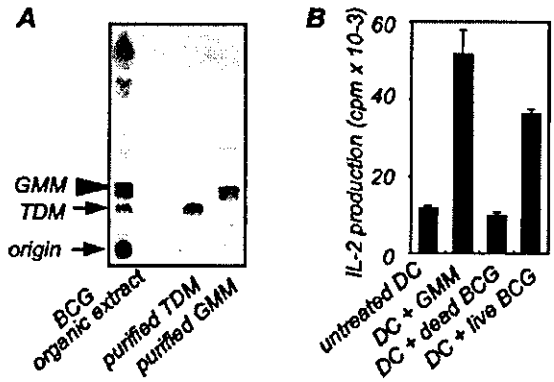

FIGURE 1. Activation of J.RT3/LDN5 cells by DCs infected with live BCG. *A*, The BCG organic extract as well as purified trehalose 6,6′-dimycolate (TDM) and GMM were resolved on silica TLC plates. *Left*, Spots corresponding to each glycolipid. *B*, J.RT3/LDN5 T cells were incubated with DCs that were untreated, GMM pulsed, or treated with either dead or live BCG for 20 h, and the amount of IL-2 released into the supernatants was measured.

was similar to that produced in the presence of untreated DCs (Fig. 1*B*, DC + dead BCG and untreated DC, respectively). Purified GMM that was heat treated was presented to J.RT3/LDN5 cells as efficiently as non-heat-treated GMM (data not shown), ruling out the possibility that heat-killed BCG might contain denatured GMM. These observations suggested that CD1b molecules functioned efficiently in monitoring live mycobacterial infection but that they might not present all Ags equally well from dead bacteria.

A pool of peripheral blood $CD8^+$ T cells recognized live BCG in a CD1- rather than MHC-dependent manner

The importance of $CD8^+$ CTLs in efficient host defense against mycobacterial infection has been well noted in mice (3, 4). Yet, it has proved difficult to identify the CD8 T cell response in humans. Thus, we sought to compare CD1-restricted and MHC-restricted responses of T cells in human peripheral blood. We used BCG-immunized subjects and examined the ability of the peripheral blood T cells to recognize live BCG-infected APCs. Given that group 1 CD1-restricted T cells have often been detected in CD4-negative T cell populations and are capable of producing IFN-γ on antigenic stimulation (11, 20), we prepared $CD8^+$ T cell-enriched and $CD4^+$ T cell-enriched populations by negative selection with appropriate Abs and magnetic beads (Fig. 2*A*, *left* and *right*, respectively) before performing IFN-γ ELISPOT assays. As shown in Fig. 2*B*, these two T cell populations were separately analyzed in triplicate in ELISPOT assays in the presence of autologous DCs that were uninfected or infected with either dead or live BCG (DC + dead BCG and DC + live BCG, respectively), and spots representing IFN-γ-producing cells were visualized (Fig. 2*B*, *top*) and counted (Fig. 2*B*, *bottom*). Whereas only a small number of $CD8^+$ T cells showed reactivity to DCs infected with dead BCG, a much larger pool was detected for live BCG-reactive $CD8^+$T cells (Fig. 2*B*, *left*). For comparison, the $CD4^+$ T cell population from the same subject was also analyzed for its reactivity to dead and live BCG (Fig. 2*B*, *right*). In contrast to the $CD8^+$T cell population that preferentially recognized live BCG, the $CD4^+$ T cell population responded better to dead BCG than to live BCG.

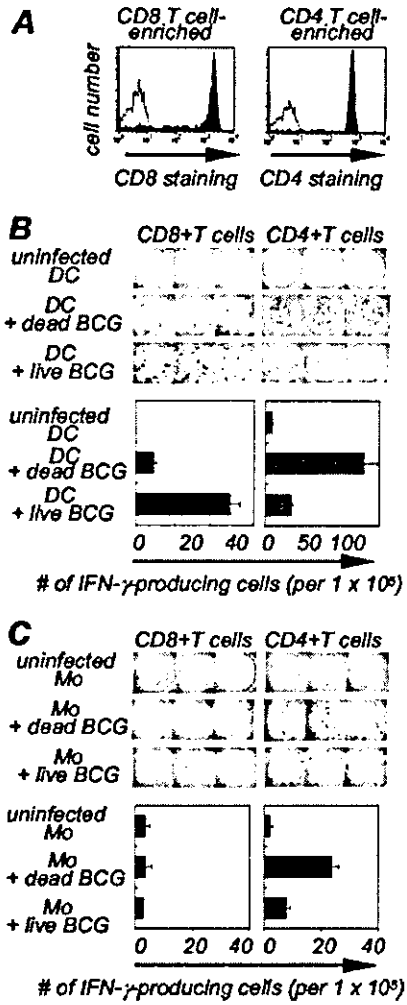

FIGURE 2. Detection of a circulating pool of live BCG-reactive $CD8^+$ T cells. *A*, The CD8 T cell-enriched (*left*) and the CD4 T cell-enriched (*right*) populations were isolated from donor 1 by negative selection, labeled with anti-CD8 and anti-CD4 Abs, respectively, and analyzed by flow cytometry (filled area). Open area, negative control staining. *B*, ELISPOT assays were performed in triplicate, using $CD8^+$ T cells and $CD4^+$ T cells as responder cells and autologous DCs that were uninfected or infected with either dead or live BCG as APCs. Spots representing IFN-γ-producing cells were visualized (*upper panels*). The spots were counted, and the mean values plus SD are shown (*lower panels*). *C*, Similar ELISPOT assays were performed, using autologous monocytes (Mo). Specific $CD8^+$ T cell activation occurred in the presence of live BCG-infected DCs, but not monocytes.

Besides DCs, macrophages are a well-known reservoir for mycobacteria (7). Unlike DCs that express both MHC and CD1 molecules, macrophages express MHC molecules prominently but lack the expression of group 1 CD1 molecules, and thus often serve as convenient APCs to analyze MHC-dependent Ag presentation pathways separately from those mediated by CD1. Therefore, we tested whether the specific $CD8^+$ T cell response to live BCG observed above with DCs might also be detected in the presence of autologous monocyte-derived macrophages. Strikingly, these CD1-negative APCs failed to activate BCG-specific $CD8^+$ T cells (Fig. 2*C*, *left*) but were capable of stimulating specific $CD4^+$ T cells, albeit less prominently than DCs (Fig. 2*C*, *right*). Thus, it seemed likely that the BCG-specific $CD8^+$ and the $CD4^+$ T cell responses might be differentially mediated by CD1 and MHC class II molecules, respectively.

To clearly determine restriction elements that were involved in live BCG recognition by these two T cell populations, ELISPOT assays with live BCG-infected DCs were performed in the presence of blocking Abs against MHC and CD1 molecules, and the percent inhibition for each Ab was calculated. Whereas the $CD4^+$T cell response to live BCG was blocked solely by anti-MHC class II Ab (Fig. 3*A*, *right*), the $CD8^+$ T cell response to live BCG was blocked most significantly by anti-CD1b Ab and moderately by anti-CD1a and anti-CD1c Abs, but not by Abs to MHC molecules (Fig. 3*A*, *left*) and anti-CD1d Abs (data not shown). Significant blockade of the $CD8^+$ T cell response to live BCG with anti-CD1 Abs ($p < 0.05$ in F test), but not by anti-MHC Abs, was observed in all 11 individuals thus far examined (Fig. 3*B*). Thus, these studies detect a circulating pool of CD1-restricted $CD8^+$ T cells that potentially monitor live mycobacterial infection.

Discussion

Live mycobacteria exhibit an outstanding ability to survive and replicate in phagosomes by inhibiting phagosomal acidification and phagosome-lysosome fusion. MHC class I and II molecules function inefficiently in mycobacteria-infected cells, because mycobacteria evasion mechanisms render protein Ags produced in phagosomes unlikely to gain easy access to the cytosol for MHC class I presentation and the lysosome for MHC class II presentation (21). In contrast, lipid and glycolipid Ags released from phagocytosed mycobacteria are often inserted into the phagosomal membrane and then traffic out of the phagosome (22). These lipids travel throughout the endocytic system of the

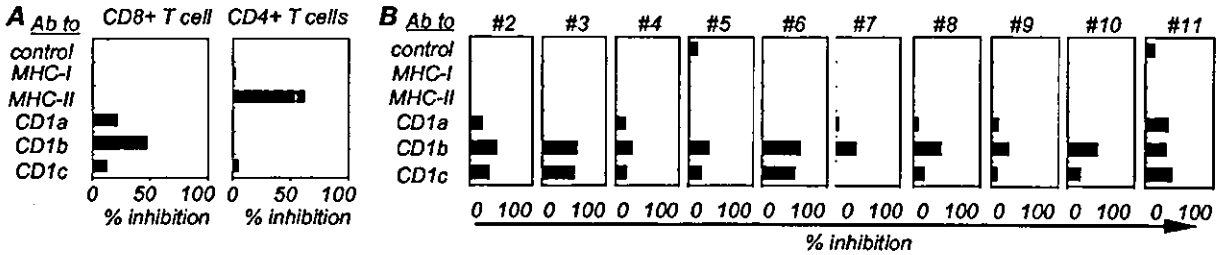

FIGURE 3. CD1-dependent recognition of live BCG by $CD8^+$ T cells. *A*, IFN-γ ELISPOT was performed in the presence of indicated blocking Abs, using T cells from donor 1 as responders and live BCG-infected autologous DCs as APCs. Blocking effect of each Ab was expressed as percent inhibition for the $CD8^+$ (*left*) and the $CD4^+$ (*right*) T cell populations. The mean numbers of positive spots for $CD8^+$ and $CD4^+$ T cells before Ab inhibition were 32 and 28, respectively. *B*, Similar Ab blocking analysis in ELISPOT assays was performed for $CD8^+$ T cells obtained from 10 different donors (donors 2–11) in the presence of live BCG-infected autologous DCs, and the blocking effect of each Ab was expressed as percent inhibition. The mean numbers of positive spots before Ab inhibition were 40.6, 24.6, 71.3, 13, 22, 24.6, 10.3, 21, 49.6, and 15 (from donors 2 through 11).

host cell, including early recycling endosomes and lysosomes, where CD1a and CD1b molecules traffic, respectively (9). Further, CD1c molecules are expressed broadly throughout the endocytic system (10). Thus, the intracellular trafficking of phagosome-derived lipid Ags in live mycobacteria-infected cells correlates with the endocytic distribution of CD1 molecules, enabling monitoring of live mycobacterial infection by the CD1 system.

In contrast, the intracellular delivery of phagocytosed dead mycobacteria is different from that for live mycobacteria, resulting in distinct pathways for Ag processing and presentation. Phagocytosed dead mycobacteria reside in phagosomes only transiently and are quickly delivered to the acidic, proteolytically active lysosomal compartments, where MHC class II molecules readily bind processed peptide Ags. The cell wall structure of dead mycobacteria that contains CD1-restricted lipid Ags also reaches the lysosome where CD1b and CD1c molecules are expressed; yet the rigid cell wall packed densely with extremely hydrophobic lipid molecules that are either covalently or noncovalently attached to each other and to the underlying arabinogalactan and peptidoglycan multilayers is hard to digest to release individual lipid components in a form that is readily sampled by CD1 molecules. Thus, the intracellular availability and distinct pathways for lipid and protein Ag processing and trafficking are likely to account for differential recognition of live and dead mycobacteria by CD1 and MHC class II molecules.

In contrast to the results presented here, previous studies have failed to detect mycobacteria-specific, CD1-restricted $CD8^+$ T cells in the peripheral blood of PPD^+ individuals (12, 13). These individuals may have had positive PPD test conversion due to natural infection with certain mycobacteria species, whereas the individuals analyzed in the present study had positive skin test conversion due to percutaneous BCG vaccine inoculation. Because alveolar mucosa and skin contain distinct subsets of macrophages and DCs, differences in the route of infection may significantly affect the quality and magnitude of T cell responses induced.

Most mycobacterial lipid-specific, CD1-restricted T cell lines thus far established are $CD4^-$, and their potential ability to control mycobacterial infection has been demonstrated in vitro, especially for $CD8^+$ T cells that produce granulysin, a bactericidal protein that directly kills mycobacteria (23). These CD1-restricted T cells notably show broad reactivity to a variety of mycobacteria species (19), contrasting MHC-restricted T cells that are highly specific and often lose reactivity when single amino acid mutation is introduced into the specific peptide Ag. Further, recent evidence has suggested that CD1-dependent lipid Ag presentation may play a crucial role in the early stages of host defense by interacting with immature DCs, even before peptide Ag-specific T cells are activated and fully expand (15, 24). Thus, the ability of CD1 to monitor infection with a broad spectrum of live mycobacteria may provide the immune system with an efficient way for the early defense against invading mycobacteria. The detection of a significant circulating pool of live mycobacteria-reactive, CD1-restricted T cells in previously infected donors emphasizes the possibility that lipid reactive T cells may contribute to host defense against mycobacterial infection.

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Predominant cell-mediated immunity in the oral mucosa: gene gun-based vaccination against infectious diseases

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Summary ***Background:*** Direct immunization via epithelial surfaces has been considered for many vaccine approaches, including DNA vaccines. It remains to be determined, however, which body site is suitable for genetic vaccination. ***Objective:*** To characterize the effects of the oral mucosa-mediated genetic vaccination, we compared antigen-specific immune responses of the oral mucosal DNA vaccine to the flank skin vaccination against influenza virus and malaria parasite. ***Methods:*** DNA vaccines against the influenza A/WSN/33 (H1N1) hemagglutinin and the malaria *Plasmodium berghei* circumsporozoite protein were administered respectively three times at 3-week intervals into the oral mucosa, skin, or liver of hamsters. The effects of their vaccine were evaluated by antigen-specific antibody production and cell-mediated killing activity. Furthermore, the in vivo malaria challenge test was also performed after the vaccination. ***Results:*** Significant specific antibody production was not observed in each case, but interferon-gamma production and cell-mediated killing activity were strongly induced in splenic lymphocytes from hamsters with the oral vaccination. The in vivo malaria challenge after the oral mucosal vaccination significantly delayed the blood-appearance day of the parasites in comparison with other immunization sites ($P < 0.05$). ***Conclusion:*** These results suggest that gene immunization via the oral mucosa may induce cell-mediated immunity more efficiently than via the skin or liver, and that the oral mucosa may be one of the

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