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Induction of Cytotoxic T Lymphocytes against the *Plasmodium falciparum* Circumsporozoite Protein by Immunization with Soluble Recombinant Protein without Adjuvant

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Immunization of mice with irradiated malaria sporozoites induces protection that is dependent on CD8$^+$ T cells, and adoptive transfer of CD8$^+$ cytotoxic T lymphocyte (CTL) clones against rodent malaria circumsporozoite (CS) protein and sporozoite surface protein 2 completely protects against sporozoite challenge. Thus, there are now efforts to develop vaccines that induce CTL against the CS protein and sporozoite surface protein 2. Until recently, it was thought that induction of CTL required production of target proteins within cells, breakdown of the proteins to peptides in the cytoplasm, and transport of the peptides to the cell surface in combination with class I major histocompatibility complex molecules. It has now been shown that immunization with peptides in Freund's complete adjuvant and with soluble protein in liposomes can induce CTL. To determine whether we could induce CTL against the *Plasmodium falciparum* CS protein by immunization with soluble protein, B10.BR mice were immunized intravenously, intraperitoneally, or intramuscularly with a recombinant *P. falciparum* CS protein called RLF mixed with the adjuvant DETOX (monophosphoryl lipid A, cell wall skeleton of *Mycobacteria phlei*, and squalane). Two weeks after the last dose, spleen cells from mice immunized intravenously, but not intraperitoneally or intramuscularly, had peptide-specific, major histocompatibility complex-restricted, CD8$^+$ T-cell-dependent cytolytic activity against peptide 368-390 from the 7G8 *P. falciparum* CS protein. To determine whether the adjuvant was required for induction of the cytolytic activity, mice were immunized with RLF without adjuvant, and similar cytolytic activity was demonstrated. The finding that we could induce CTL by administration of soluble protein without adjuvant markedly broadens the possibilities for vaccinologists working to develop methods of inducing CTL in humans.

Immunization of mice with a recombinant *Salmonella* sp. expressing the *Plasmodium berghei* circumsporozoite (CS) protein (1) and transfected P815 mouse mastocytoma cells expressing the *P. yoelii* CS protein or *P. yoelii* sporozoite surface protein 2 (7) partially protects against malaria. This protective immunity is eliminated by in vivo depletion of CD8$^+$ T cells, indicating that cytotoxic T lymphocytes (CTL) against these proteins are required for this protection. Furthermore, adoptive transfer of CTL clones against the *P. berghei* (14) and *P. yoelii* CS proteins (13, 17) can completely protect against sporozoite challenge, indicating that CTL can protect in the absence of other parasite-specific immune responses. Accordingly, some current human vaccine development efforts are directed towards induction of CTL against the *P. falciparum* CS protein and sporozoite surface protein 2.

Induction of CTL has generally been thought to require transport of peptides from the cytosol into the lumen of the endoplasmic reticulum, where the peptides bind to major histocompatibility complex (MHC) class I heavy chains and are assembled with β_2-microglobulin. The bound peptides are transported to the cell surface with MHC class I molecules, where they can be recognized by T cells. Until recently, it was thought that the proteins had to be produced inside the cells for them to enter the class I MHC pathway effectively and that this would require recombinant live vector vaccines such as a recombinant *Salmonella* sp. (1), vaccinia virus (8), or pseudorabies virus (15). However, in the past few years it has been demonstrated that immunizing with short lipopeptides (2), ISCOMS (16), and peptides in Freund's complete adjuvant could all induce CTL (11). Recently, it has been demonstrated that CTL can be induced by immunization with soluble protein in liposomes (10).

We have conducted clinical studies with a malaria protein administered with an adjuvant containing monophosphoryl lipid A (MPL), cell wall skeleton (CWS) mycobacteria, and squalane (12). When compared with aluminum hydroxide, this adjuvant (DETOX) improved the antibody response against the malaria protein (12). The current studies were undertaken to determine whether immunization of mice with a recombinant malaria protein in this adjuvant would induce CTL. Having established that this was the case, we demonstrated that immunization with this soluble recombinant malaria protein induces CTL even without the use of adjuvant.

MATERIALS AND METHODS

Mice. Six-week-old B10.BR (H-2^k) mice were purchased from Jackson Laboratories, Bar Harbor, Maine. The experiments reported herein were conducted according to the principles set forth in the *Guide for the Care and Use of Laboratory Animals*.

Immunogen. The CS protein of the 7G8 clone of the *P. falciparum* CS protein includes 412 amino acids. It has a

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central repeat region with 37 copies of NANP and 4 copies of NVDP, a total of 164 amino acids. A recombinant protein that included the entire *P. falciparum* CS protein minus these central 164 amino acids was produced, fused to 81 amino acids from the nonstructural protein of influenza A virus attached at the carboxyl end, and purified so that it could be used to immunize humans. The formulation was greater than 90% pure and met Food and Drug Administration standards for endotoxin levels and DNA content (5). This repeatless CS protein is called RLF (SB Pharmaceuticals, King of Prussia, Pa.).

Adjuvant. The adjuvant used was MPL-CWS (DETOX; Ribi ImmunoChem Research Inc., Hamilton, Mont.). It includes CWS of *Mycobacterium phlei*, MPL, and squalane (12).

Immunization. B10.BR mice were immunized by the intraperitoneal, intramuscular, or intravenous (i.v.) route with the following combinations in 0.2 ml: (i) for DETOX, 100 μg of CWS, 10 μg of MPL, and 1% squalane; (ii) for RLF, 50 μg of RLF; and (iii) for RLF-DETOX, 100 μg of CWS, 10 μg of MPL, 1% squalane, and 30 μg of RLF.

Recombinant vaccinia virus. V71, a recombinant vaccinia virus containing the CS gene of *P. falciparum*, has been described before (8).

Peptides. Only B10.BR (*H-2^k*) mice have been shown to produce CTL against the *P. falciparum* CS protein, and in B10.BR mice only amino acids 368 to 390 have been shown to include a CTL epitope (8). This region has also been shown to include a CTL epitope for humans (9). A 23-amino-acid synthetic peptide (KPKDELDYENDIEKKICKMEK CS), including amino acid residues 368 to 390 (Pf CSP 368-390), and a 20-amino-acid control peptide [Pf CS $(PNAN)_5$; amino acids 151 to 170], including five copies of the major repeat of the *P. falciparum* CS protein, were used (9).

Effector cells. Mice in each group had received two immunizations. Two weeks after the last immunization, spleen cells were isolated for use as effector cells. Briefly, 5×10^6 spleen cells were plated in 24-well tissue culture plates in a final volume of 2 ml of complete medium (RPMI 1640 medium supplemented with 10% fetal calf serum, L-glutamine [2 mM], 2-mercaptoethanol [5×10^{-5} M], and penicillin and streptomycin [50 U/ml each]).

Stimulator cells. L cells (*H-2^k*) transfected with the DNA encoding the full-length *P. falciparum* CS protein (8) were used for in vitro stimulation. The transfected L cells (LPf) were treated with mitomycin (50 μg/ml), and then 2×10^5 LPf per well were added to 24-well plates that contained 5×10^6 effector cells per well. Cultures were then incubated for 5 days at 37°C in 5% CO_2.

Target cells. LPf (*H-2^k*), untransfected L cells (*H-2^k*), and P815 mouse mastocytoma cells (*H-2^d*) were used as target cells. Eighteen hours before a CTL assay, 10^6 L cells or P815 cells were incubated in the presence of 100 μg of peptide per ml and 100 μCi of sodium chromate (Dupont-New England Nuclear Inc., Boston, Mass.) in 2 ml of medium. LPf, 10^6, were incubated in 2 ml of medium with 100 μCi of sodium chromate only.

Depletion of T lymphocytes. In vitro depletion of $CD4^+$ and $CD8^+$ T lymphocytes from the effector cells was done with anti-CD4 monoclonal antibody GK 1.5 (rat immunoglobulin G subclass 2b) (3) and anti-CD8 monoclonal antibody 19/178 (mouse immunoglobulin G subclass 2a) (6). Briefly, effector cells were incubated with anti-CD4 or anti-CD8 monoclonal antibodies for 30 min. Freshly prepared complement (Low-Tox rabbit complement; Cedarlane Laboratories, Hornby, Ontario, Canada) was added at a final concentration of 10%. After 30 min of incubation at 37°C, cells were washed twice in RPMI 1640 medium (GIBCO, Grand Island, N.Y.) and then used in a chromium release assay.

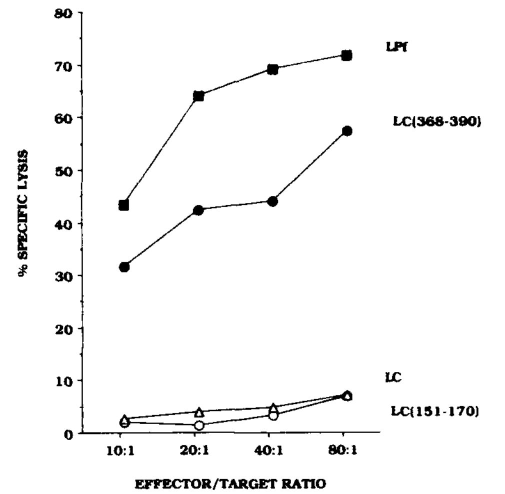

FIG. 1. CTL activity against the *P. falciparum* CS protein (peptide 368-390) after i.v. immunization of B10.BR mice (*H-2^k*) with 10^7 PFU of recombinant vaccinia virus expressing the *P. falciparum* CS protein. One month later, spleen cells (pool of two spleens) were stimulated for 6 days in vitro with L cells expressing the *P. falciparum* CS protein (LPf). These effectors lysed L cells pulsed with peptide 368-390 [LC(368-390)] (●) and LPf (■) but did not lyse L cells pulsed with control peptide 151-170 [LC(151-170)] (△) or L cells (LC) not exposed to peptide (○).

Chromium release assay. For in vitro cytotoxicity assays, 5,000 chromium-labelled target cells were incubated with effector cells for 6 h in a final volume of 0.2 ml in 96-well round-bottom plates (Costar, Cambridge, Mass.) in triplicate. Supernatants were harvested by using a Skatron SCS system (Skatron Inc., Sterling, Va.), and chromium-51 released in supernatants was measured with a gamma counter (Clinnigamma; Pharmacia LKB Nuclear Inc., Gaithersburg, Md.). Maximum chromium-51 release was determined by lysing target cells with 5% Triton X-100. Percent specific lysis was determined as follows: 100 × [(experimental release − spontaneous release)/(maximum release − spontaneous release)].

RESULTS

Immunization of mice with RLF-DETOX induces CTL activity. To establish a baseline for the assays, B10.BR mice were immunized with a recombinant vaccinia virus expressing the *P. falciparum* CS protein (8). The spleen cells were stimulated with LPf, and either chromium-labelled LPf or peptide-pulsed, chromium-labelled L cells were used as targets. LPf were somewhat better as targets than were peptide Pf CSP 368-390-pulsed targets, but both were good targets for CTL (Fig. 1). When B10.BR mice were immunized with two doses of RLF-DETOX intraperitoneally or intramuscularly no CTL could be identified (data not shown). When the mice were immunized with two i.v. doses of RLF-DETOX, CTL against peptide 368-390 were identified (Fig. 2a). This response was antigen specific, MHC restricted, and, like classical CTL, dependent on $CD8^+$ T cells (Fig. 2a and b). When mice were immunized i.v. with

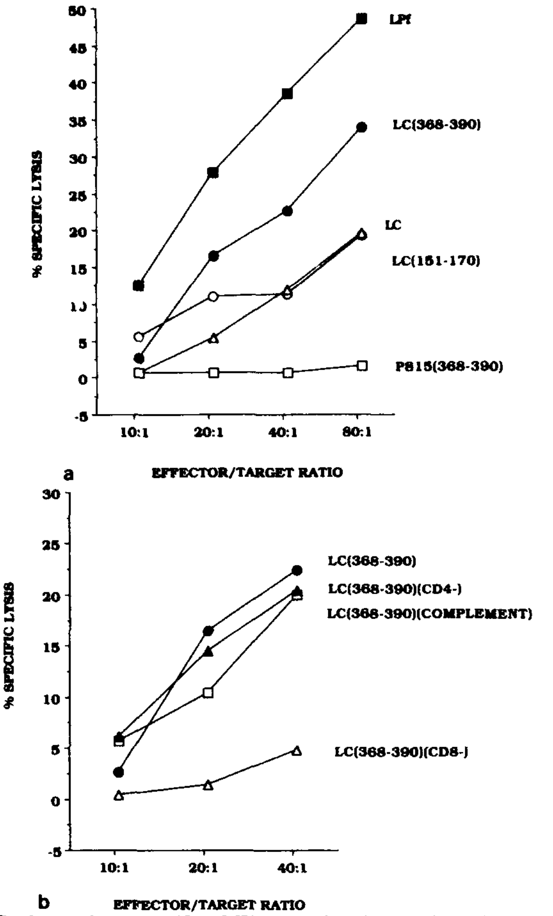

FIG. 2. Antigen-specific, MHC-restricted, CD8$^+$ CTL activity after i.v. immunization of B10.BR mice with RLF-DETOX. (a) Spleen cells (pool of two spleens) were stimulated in vitro with LPf for 6 days. These effectors lysed L cells (H-2^k) pulsed with peptide 368-390 (●) and LPf (■) but not L cells pulsed with peptide 151-170 (△), L cells not exposed to peptide (○), or P815 cells (H-2^d) pulsed with peptide 368-390 [P815(368-390)] (□). (b) Spleen cells stimulated for 1 week with LPf were depleted of CD8$^+$ T cells (△) or CD4$^+$ T cells (▲) or just treated with complement alone (□). Only CD8$^+$ T-cell depletion reversed the cytolytic activity.

DETOX alone and their spleen cells were stimulated in vitro with LPf in the same manner as the cells from the mice immunized with RLF-DETOX, no CTL activity could be demonstrated (Fig. 3). Experiments in each group were done at least two times.

Induction of CTL against the *P. falciparum* CS protein by immunization with soluble protein alone. Having established that mice immunized with the adjuvant-protein mixture, RLF-DETOX, produced CTL against the same epitope as mice immunized with a recombinant live vaccinia virus that expressed the CS protein (8), we wondered whether the adjuvant was actually necessary for the induction of CTL. We therefore immunized mice with two i.v. doses of RLF without adjuvant. Spleen cells were stimulated with LPf, and a CTL assay was performed with LPf targets and peptide-pulsed targets. The effector cells lysed L cells pulsed with peptide Pf CSP 368-390 but did not lyse P815 cells pulsed with the same peptide or L cells pulsed with peptide Pf CS (PNAN)$_5$ (Fig. 4). As in the previous experiments, percent specific lysis of LPf targets was greater than percent specific

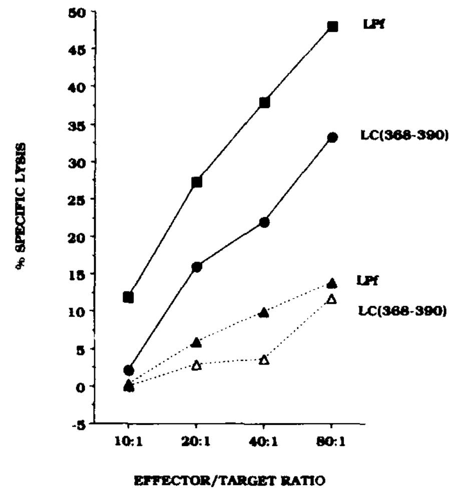

FIG. 3. Inability of i.v. immunization with adjuvant (DETOX) to induce CTL. Spleen cells (pool of two spleens) from mice immunized with adjuvant alone and stimulated with LPf did not lyse LPf targets (▲) or L cells pulsed with peptide 368-390 (△). In contrast, cells from mice immunized with RLF-DETOX lysed LPf (■) and L cells pulsed with peptide 368-390 (●).

lysis of peptide Pf CSP 368-390-pulsed targets at effector/target ratios of 80:1 (47.5% ± 2.2% versus 26.1% ± 0.6%, mean ± standard error of the mean; P = 0.012, Student's t test, two-tailed), 40:1 (39.2% ± 1.5% versus 19.1% ± 0.6%; P = 0.006), and 20:1 (26.0% ± 1.3% versus 10.5% ± 1.6%; P = 0.005).

DISCUSSION

Immunization of mice and humans with radiation-attenuated malaria sporozoites induces solid protective immunity against sporozoite challenge. In a number of strains of mice,

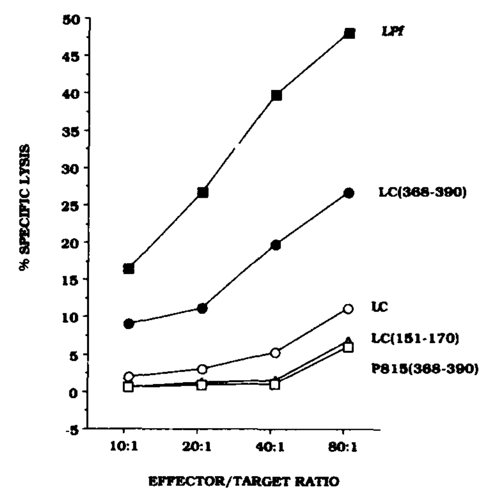

FIG. 4. Antigen-specific, MHC-restricted cytolytic activity after i.v. immunization with RLF without adjuvant. Spleen cells (pool of two spleens) from B10.BR mice immunized i.v. with two doses of RLF and stimulated for 6 days in vitro with LPf lysed L cells pulsed with peptide 368-390 (●) and LPf targets (■) but did not lyse L cells pulsed with peptide 151-170 (△), L cells not exposed to any peptide (○), or P815 (H-2^d) cells pulsed with peptide 368-390 (□).

this immunity is dependent on CD8$^+$ T cells, and CD8$^+$ CTL clones against the CS protein and sporozoite surface protein 2 adoptively transfer protection against malaria. Thus, there has been considerable work aimed at developing subunit vaccines that induce comparable protective CTL. This work has primarily focused on the use of live recombinant vectors.

However, the storage, delivery, and fielding, and in some cases even the production, of such vaccines would be much more difficult than would those of a vaccine that relied on synthetic peptide or recombinant protein. Work with peptides (2, 11, 16), which are by necessity limited in size to a small portion of any protein, and most recently with soluble protein in liposomes (10) has clearly demonstrated that induction of CTL does not require the production of protein within the cell. The mechanism(s) by which CTL have been induced in those studies has not been established. It has been proposed that the liposomes facilitate the delivery of the protein within the cells, where some escapes into the cytosol and is broken down to peptides, which then enter the class I MHC pathway like peptides from protein produced within the cell (10). In the case of peptides, the peptide may be delivered to the cytosol, or the peptide may complex with class I MHC on the surface of the cell, without ever entering the cytosol.

Incorporation of protein and adjuvant into liposomes requires special expertise. The mixture of complete Freund's adjuvant with a peptide induces CTL (11) but cannot be given to humans. The adjuvant DETOX was designed to produce immune responses comparable to those induced by complete Freund's adjuvant. CWS of *M. phlei* substitutes for the mycobacteria and squalane substitutes for the mineral oil in complete Freund's adjuvant. MPL is added as an additional immunopotentiator. The data presented in Fig. 2 and 3 indicated that administration of RLF with this adjuvant induced CTL against a specific epitope on the *P. falciparum* CS protein. However, when we immunized mice with the recombinant fusion protein alone without adjuvant, we found comparable induction of CTL (Fig. 4). We have no data to support a hypothesis for why this protein is able to induce CTL against the *P. falciparum* CS protein without adjuvant. It is possible that the protein was partially degraded and that we actually immunized the mice with peptide Pf CSP 368-390 that bound directly to class I MHC on the cell surface and somehow bypassed the requirement for internal protein processing and export to the cell surface. Another explanation is that the structure of the fusion protein, RLF, is unique and that this structure promotes entry of the protein into the cell, where it is broken down in the cytoplasm. The uniqueness could be related to the structure of the malaria component of the fusion protein or due to the 81 amino acids from the nonstructural protein of influenza A virus. The recent demonstration of induction of CTL against influenza virus by immunization with a recombinant influenza virus hemagglutin subunit protein linked to the same 81 amino acids from the nonstructural protein of influenza A virus, and delivered with aluminum hydroxide as an adjuvant (4), suggests that these 81 amino acids facilitate the induction of CTL against the CS protein and influenza virus.

Work is in progress to characterize the steps involved in induction of these CTL and to determine why i.v., but not intramuscular or intraperitoneal, immunization was successful in inducing CTL in our experiments. We are also investigating whether the increased lysis of LPf compared with peptide-pulsed targets was due to recognition of additional CTL epitopes on the full-length Pf CS protein in mice immunized with RLF. Regardless of the outcome of these studies, our data demonstrate the induction of CTL against the *P. falciparum* CS protein by immunization with soluble protein without adjuvant. Since *P. falciparum* does not infect mice, we cannot study the protective activity of these CTL. Nonetheless, this finding further opens the possibility for the development of soluble protein vaccines designed to induce CTL against specific proteins.

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