

# The In Vivo Cytotoxic Activity of CD8<sup>+</sup> T Cell Clones Correlates with Their Levels of Expression of Adhesion Molecules

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## Summary

CD8<sup>+</sup> T cell clones specific for a defined epitope present in the circumsporozoite protein of *Plasmodium yoelii* display striking differences in their in vivo antiplasmodial activity. The adoptive transfer of certain clones (YA23 and YA26) into naive mice inhibits by 90% or more the development of liver stages of malaria parasites and protects against malaria infection. The adoptive transfer of two other T cell clones (YB8 and YA15) results, respectively, in partial or no inhibitory activity on parasite development. We found that "protective" and "nonprotective" cytotoxic T lymphocyte (CTL) clones do not differ in their fine epitope specificity and display similar levels of lysis and DNA degradation of target cells in vitro. Their pattern of production of lymphokines and granule-associated proteins also failed to correlate with their in vivo antiplasmodial activity. Histological studies combined with autoradiography showed that, upon adoptive transfer, only T cells from the protective CTL clones are capable of "associating" with a significant percentage of parasitized hepatocytes. Fluorescence-activated cell sorter analysis of surface molecules revealed pronounced differences in the levels of CD44 and VLA-4 expression by the different clones, correlating closely with their in vivo protective activity. The correlation between in vivo antiparasite activity and the expression of CD44 was further corroborated by the results of sorting, from the partially protective YB8 clone, two sub-populations expressing high and low levels of CD44. These were protective and nonprotective, respectively. The clones also differed in their adhesive properties. Cross-linking of CD44, using specific antibodies, induced LFA-1-mediated homotypic aggregation of protective clones, while nonprotective cells failed to aggregate.

A number of recent experimental findings have provided evidence that CD8<sup>+</sup> T cells, induced by immunization with radiation-attenuated sporozoites, play an important role in immunity against the liver stages of malaria parasites. Recently, we obtained CD8<sup>+</sup> T cell clones directed against defined epitopes present in the circumsporozoite (CS)<sup>1</sup> proteins of two rodent malaria species, *Plasmodium berghei* and *P. yoelii* (1, 2). Some of these clones inhibit the development of the intrahepatocytic stages of the corresponding malaria parasites when adoptively transferred to naive mice and protect these mice against malaria infection.

A surprising finding of these studies was that different

CD8<sup>+</sup> T cell clones displayed striking differences with regard to their in vivo antiplasmodial activity. Using a quantitative method, based on the measurement of plasmodial ribosomal RNA in the liver of *P. yoelii*-infected mice, we compared the in vivo antiparasite activity of various CD8<sup>+</sup> T cell clones. We found that the development of *P. yoelii* liver stages could be inhibited, by 90% or more, when naive mice were injected with the T cell clones YA26 or YA23, and by 50–60% when the mice received the YB8 T cell clone. In contrast, the adoptive transfer of T cell clone YA15 had no detectable antiparasite effect, although clones YA23, YA26, and YA15 were derived in the course of the same cloning procedure from the spleen cells of a single mouse immunized with sporozoites (2).

To elucidate the basis of the functional heterogeneity displayed by these T cell clones, we undertook detailed comparative studies aimed at characterizing their fine epitope

<sup>1</sup> Abbreviations used in this paper: CS, circumsporozoite; LT, lymphotoxin.

specificity, patterns of lymphokine production, tissue migration, and localization after adoptive transfer. The results of the present study indicate that protective and nonprotective clones differ in their capacity to associate in vivo with parasitized liver cells and that their protective capacity correlates closely with the expression of certain defined adhesion molecules.

## Materials and Methods

**Parasites and Animals.** *P. yoelii* (17X NL strain) was maintained by repeated cyclic passage of the parasites in *Anopheles stephensi* mosquitoes and BALB/c mice. Sporozoites were collected by dissecting the mosquito salivary glands in medium 199, ~2 wk after their infective blood meal.

4–8-wk-old female BALB/c mice used for the immunological studies were purchased from The Jackson Laboratories (Bar Harbor, ME) and from Charles River Laboratories (Wilmington, MA). Mice were challenged intravenously with the indicated dose of viable sporozoites.

**Cell Culture.** As culture medium, we used DME-high glucose (Grand Island Biological Co., Long Island, NY), supplemented with 0.2 g/liter of L-arginine (Calbiochem-Behring Co., La Jolla, CA), 0.036 g/liter of L-Asparagine (Gibco Laboratories, Grand Island, NY), 10 mM Hepes, 2 mM L-Glutamine, and 10% FCS (HyClone Laboratories, Logan, UT). For growth and maintenance of the CTL clones, we also added to the medium  $5 \times 10^{-5}$  M 2-ME and 2% of T cell growth factor medium, derived from PMA- (Sigma Chemical Co., St. Louis, MO) activated EL-4 cells. The cultures were maintained at 37°C in an atmosphere containing 5% CO<sub>2</sub>.

**Stimulation and Adoptive Transfer of CTL Clones.** CTL clones were established and maintained as described (2). Briefly, CTL clones ( $1.25 \times 10^5$ ) were restimulated weekly with irradiated feeder cells ( $2.5 \times 10^6$  cells/ml), and irradiated P815 cells ( $1.25 \times 10^5$  cells/ml) pulsed with 1  $\mu$ M of peptide Py 277-288. 4 d later, and after the addition of an equal volume of medium, the cultures were divided and grown under the same conditions for an additional 2–3 d. The desired number of T cells was centrifuged and resuspended in DME containing  $2 \times 10^3$  U/ml of human rIL-2 (kindly provided by Hoffmann-La Roche, Inc., Nutley, NJ), immediately before transfer. A total volume of 0.5 ml of the cell suspension or IL-2 alone was injected intravenously into each mouse. The timing of cell transfer in relation to sporozoite challenge varied from 6 h before to 24 h after parasite inoculation as specified in the figure legends.

**Quantification of *P. yoelii* rRNA in the Liver of Infected Mice.** Quantification of *P. yoelii* rRNA was performed as described (3). Briefly, total RNA was isolated from the livers of mice killed 42 h after they had been injected intravenously with  $5 \times 10^5$  sporozoites of *P. yoelii*. RNA was prepared by the method of Chomczynski and Sacchi (4). 10% of the whole liver RNA was precipitated with isopropanol. The RNA pellet was dissolved in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, denatured at 65°C in  $20 \times$  SSC ( $1 \times$  SSC is 150 mM NaCl, 15 mM Na Citrate) plus 37% formaldehyde (1:1). This preparation was diluted 45 times, and 0.2-ml samples were blotted onto nylon membranes. The RNA was fixed to the filters by UV crosslinking. Hybridization was performed overnight in  $5 \times$  SSPE ( $1 \times$  SSPE contains 180 mM NaCl, 10 mM Na phosphate, 1 mM EDTA, pH 7.7), 1% SDS, and 500  $\mu$ g heparin at 42°C, containing three oligonucleotide probes labeled with <sup>32</sup>P ( $10^6$  cpm/ml; sp act  $>2 \times 10^8$  cpm/ $\mu$ g). Filters were washed twice in  $6 \times$  SSC + 0.1% SDS and once with  $1 \times$  SSC + SDS, at room

temperature for 15 min. The membranes were dried, the spots on the filters were cut out, and the radioactivity was measured in a liquid scintillation counter. For comparison purposes, a standard curve was prepared using serial dilutions of purified RNA from *P. yoelii*-infected erythrocytes, blotted under the same conditions.

**Chromium Release Assay.** For this in vitro cytolytic assay, we used a P815 (H-2<sup>d</sup>) cell line, standardly used as a target cell, labeled with 250  $\mu$ Ci of <sup>51</sup>Cr (ICN Biomedicals, Irvine, CA). After washing,  $2 \times 10^3$  P815 cells per well were cultured with  $10^4$  CTL, in the presence of the desired concentration of peptide. After 4 h at 37°C, the supernatants were collected with the aid of a semi-automatic harvester (Skatron, Inc., Sterling, VA). The percentage of specific lysis was calculated as follows:  $100 \times (\text{experimental} - \text{spontaneous release cpm}/\text{total} - \text{spontaneous release})$ .

**DNA Degradation Assay.** The target cells were P815 cells, labeled overnight with 5  $\mu$ Ci/ml of [<sup>3</sup>H]thymidine (6.7 Ci/mM; New England Nuclear, Boston, MA). The assay was performed in 1.5-ml microfuge tubes, where target cells ( $10^4$ ) were cultured at 37°C with different ratios of CTL in the presence or absence of peptide Py 277-288 (1  $\mu$ M) in a final volume of 0.2 ml. After 2 h, each sample received 1 ml of lysis buffer (20 mM Tris, 1 mM EDTA, 0.2% Triton X-100 [vol/vol]; pH 8.0). These samples were centrifuged for 10 min at 12,000 g, and 0.6 ml of sample supernatants was collected and counted in a scintillation counter. The percentage of specific DNA degradation was calculated as follows:  $100 \times (\text{experimental} - \text{spontaneous release cpm}/\text{total} - \text{spontaneous release})$ .

**IFN- $\gamma$ , TNF/Lymphotoxin (LT), and BLT-Esterase Determination.** P815 cells were pulsed with 1  $\mu$ M of peptide Py 277-288 for 30 min before the assay.  $10^6$ /ml of these target cells were cultured with the same amount of CTL in 96-well V-bottomed plates (Costar, Cambridge, MA). The final volume was 0.2 ml, and the cultures were kept at 37°C. After 6 h of incubation, the plates were centrifuged, and the culture supernatants were collected and used to measure IFN- $\gamma$ , TNF/LT, and BLT-esterase.

The amount of IFN- $\gamma$  was determined by RIA, as described (1). Murine rIFN- $\gamma$  (Genentech, San Francisco, CA) was used as a standard.

The TNF/LT bioassay was performed as described (5). Briefly,  $3 \times 10^5$  L-929 cells were seeded in 96-well flat-bottomed culture plates (Costar). Culture supernatants from CTL, generated as described above, were added in a 0.1-ml volume at a final concentration of 50% (vol/vol). Recombinant TNF- $\alpha$  (Genentech) was used as standard. After 24 h, L cell survival was evaluated by the incorporation, in the course of four hours, of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) (Sigma Chemical Co.). The cell suspension was then acidified with 100  $\mu$ l of acid isopropanol. After 5 min, 100  $\mu$ l of water was added to each well and kept at room temperature for 1 h before reading at 470 nm. BLT-esterase activity was detected by adding 20  $\mu$ l of culture supernatants in 0.18 ml of Tris-HCl buffer (20 mM Tris, 1 mM EDTA, pH 7.5) containing 200  $\mu$ M of 5,5'-Dithio-bis (2-nitrobenzoic acid; Sigma Chemical Co.) and 220  $\mu$ M of N-benzoyloxycarbonyl L-lysine thiobenzylester (BLT; Sigma Chemical Co.). The samples were kept at room temperature for 30 min before reading at 405 nm.

**Synthetic Peptides.** A series of nested peptides containing the CTL epitope of the CS protein of *P. yoelii* was synthesized as described (6).

**Labeling of CTL.** CTLs were harvested 6–7 d after restimulation with peptide. After being washed in methionine-free RPMI (Gibco Laboratories), the CTL ( $5 \times 10^6$  cells/ml) were incubated in the same medium containing 10% of dialyzed FCS and 100  $\mu$ Ci/ml of <sup>35</sup>S-Methionine (New England Nuclear). After 2 h at

37°C, the cells were washed three times in culture medium, and adoptively transferred to BALB/c mice ( $2.0 \times 10^7$  per mouse), which had been infected with  $10^6$  sporozoites, 24 h earlier.

**Tissue Fixation, Embedding, Sections and Autoradiography.** Livers from mice injected only with parasites, or with parasites and CTL clones, were fixed overnight in PBS containing 10% (vol/vol) formalin, dehydrated through a graded series (70%, 95%, and 100%) of ethanol, 100% chloroform, and embedded in paraffin. Transverse sections of paraffin blocks were cut at 4- $\mu$ m thickness and mounted on glass slides. Deparaffination was performed by dipping slides into xylene and subsequently rehydrating them by passage through a graded series of ethanol (100%, 90%, and 70%) and finally tap water. Slides were dipped in Kodak NTB-2 emulsion (Kodak, Rochester, NY) prediluted 1:3 with distilled water, and equilibrated at 42°C. The dipped slides containing the tissue samples were dried, and the exposure in a desiccator was continued for 24–48 h at 4°C. Slides were developed in Kodak D-19, at 21°C, for 6 min, and counterstained with hematoxylin and eosin according to standard protocols.

**Flow Cytometry and Cell Sorting.**  $10^6$  T cells were incubated for 45 min, on ice, with purified antibodies, undiluted hybridoma supernatants, or hybridoma supernatants precipitated with ammonium sulfate. After being washed twice, cell samples were incubated with fluorescein-labeled goat anti-rat IgG or goat anti-hamster IgG (KPL Inc., Gaithersburg, MD), for an additional 45 min on ice, washed, and fixed in PBS containing 2% (wt/vol) of paraformaldehyde. Fluorescence was analyzed in a FACScan<sup>®</sup> cytometer (Becton Dickinson & Co., Mountain View, CA), gating for size by forward and side ward light scatter, both amplified on linear scales. The fluorescence signals were amplified on a logarithmic scale.

The following rat IgG mAbs were used for staining: anti-CD8 (2.4.3), anti-LFA-1 (FD441.8), anti-L-selectin (MEL-14), anti-ICAM-1 (YN1/1.7.4), anti-CD44 (KM 703, KM 201), anti-CD45Ra (TIB 164), all purchased from American Type Culture Collection (ATCC; Rockville, MD). Other rat mAbs used were: anti-VLA-4/LPAM-1 (R1-2; Pharmingen, San Diego, CA); anti-CD45 (M1/9.3.4 H2-2) and CD45Rb (4B4) (a kind gift from Dr. E. Pure, The Rockefeller University, New York); anti-V $\beta$  8.1 and 8.2 (KJ16), anti-V $\beta$  8.2 (F23.2), and anti-CD44 (IM7.8.1) (kindly provided by Dr. H. R. McDonald, Ludwig Institute for Cancer Research, Lausanne

Branch, Switzerland); anti-CD2 (RM2-2) kindly provided by Dr. A. Sen, Dana-Farber Cancer Institute, Boston, MA). The hamster IgG mAb to the vitronectin receptor (H9-2B8) was kindly provided by Dr. E. Shevach (NIAID, Bethesda, MD). For cell sorting, we used an EPIC 752 (Coulter Electronics Inc., Hialeah, FL).

**Aggregation Assay.** CTL cells were harvested 7 d after restimulation with irradiated feeder cells and irradiated P815 target cells coated with peptide. These cells were washed five times in culture medium to eliminate dead cells and cell debris. The cell suspension obtained showed 99% or more viability and was seeded in 96-well flat-bottomed plates (Dinatech, Chantilly, VA). Each well contained  $1.5 \times 10^5$  T cells. To these cells we added two mAbs to CD44 (KM 201 and KM 703), at a final concentration of 5  $\mu$ g/ml each, or 100 ng/ml of PMA, in a final volume of 0.2 ml. Some of the wells received 25% of a supernatant containing anti-LFA-1 mAb. The plate was then centrifuged for 2 min and incubated at 37°C for 2 h. Cultures were photographed as viewed in an inverted phase contrast microscope.

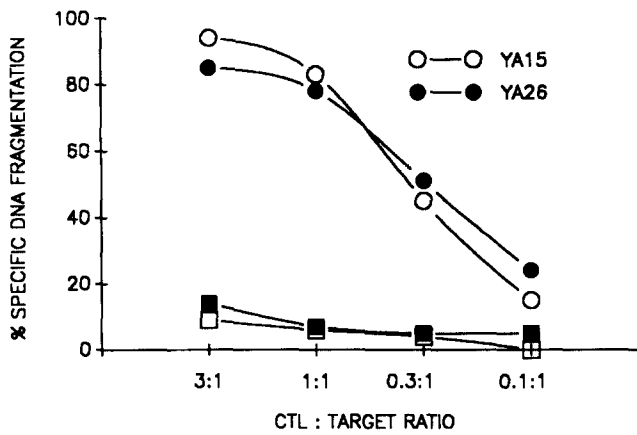
## Results

**Protective and Nonprotective CD8<sup>+</sup> CTL Clones, Fine Epitope Specificity, and In Vitro Activities.** As previously described, protective and nonprotective CD8<sup>+</sup> T cell clones display similar levels of in vitro cytolytic activity when incubated with target cells coated with the 12mer synthetic peptide, representing amino acids 277–288 of the *P. yoelii* CS protein (2). To further characterize the fine epitope specificity of the protective YA26 and nonprotective YA15 clones, cytolytic assays were performed with a series of nested peptides. All these peptides contained the minimal amino acid sequence, comprised between the tyrosine and isoleucine residues, essential for the binding of peptides to H-2K<sup>d</sup> MHC molecules (6–8). As seen in Table 1, the two T cell clones do not differ significantly in their capacity to recognize these different synthetic peptides, since both clones require similar peptide concentrations to induce 50% of maximal lysis of target cells. Both clones were most efficient when target cells were coated

**Table 1.** Recognition of NH<sub>2</sub>-terminally Truncated Synthetic Peptides Containing the *P. yoelii* Cytotoxic Epitope, by a Protective (YA26) and a Nonprotective (YA15) CTL Clone

Peptide	Sequence	Peptide concentration necessary for 50% maximal lysis	
		YA26	YA15
		<i>M</i>	
277–288	NEDSYVPSAEQI	$5.5 \times 10^{-10}$	$2.5 \times 10^{-10}$
278–288	EDSYVPSAEQI	$3.3 \times 10^{-10}$	$3.5 \times 10^{-10}$
279–288	DSYVPSAEQI	$9.0 \times 10^{-11}$	$1.0 \times 10^{-10}$
280–288	SYVPSAEQI	$5.0 \times 10^{-12}$	$2.2 \times 10^{-12}$
281–288	YVPSAEQI	$2.5 \times 10^{-11}$	$1.6 \times 10^{-11}$

Chromium-labeled P815 cells were incubated with various peptides at different concentrations, together with YA26 or YA15 CTLs, at a 5:1 E/T ratio. The results are expressed as the molar concentration of peptide required to obtain 50% lysis of target cells. The maximal specific lysis obtained was 66% and 76%, using YA26 and YA15 clone, respectively.



**Figure 1.** Protective (YA26) and nonprotective (YA15) CTL clones induce similar levels of DNA degradation of peptide-coated P815 cells. The CTL of both clones were incubated with target cells (squares), or target cells that had been coated with 1  $\mu$ M of peptide Py 277-288 (circles).

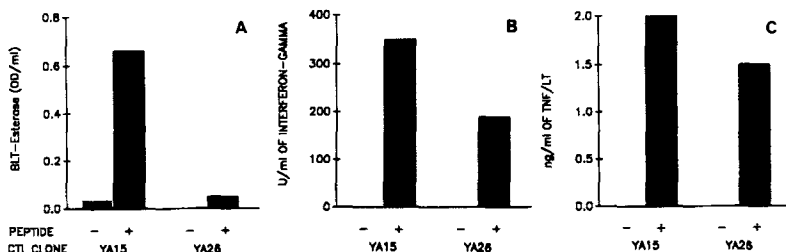
with the 9mer peptide, i.e., the peptide that binds more efficiently to the H-2K<sup>d</sup> molecule (6, 7).

In addition to lysis, these clones also induce DNA degradation in target cells. As shown in Fig. 1, both T cell clones (YA15 and YA26) have almost identical capacity to degrade the DNA of peptide-coated P815 target cells.

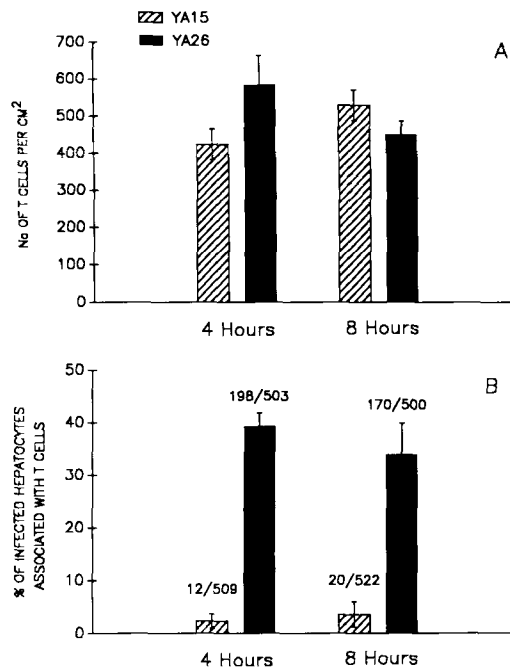
The respective levels of granule-associated proteins believed to be involved in the cytolytic event also do not correlate with the observed differences in the antiparasite activity of these two clones. The quantification of perforin in cell extracts of both clones, using a hemolytic assay, as well as the levels of BLT-esterase, consistently revealed that nonprotective YA15 cells have greater perforin activity (data not shown) and produce much larger amounts of BLT-esterase than YA26 cells (Fig. 2 A).

Because IFN- $\gamma$  and TNF/LT can inhibit the development of liver stages of malaria parasites (9, 10), we measured the production of these cytokines by these two clones after peptide stimulation. As shown in Fig. 2, B and C, no significant differences were observed in the capacity of these two clones to produce IFN- $\gamma$  and TNF/LT.

*Migration of Protective and Nonprotective Clones to the Liver and within Its Parenchyma.* Since the liver stages are the target of protective CTL, a failure to migrate to the liver could explain the inability of YA15 cells to eliminate malaria parasites. To study this question, we analyzed the circulation pat-



**Figure 2.** Secretion of BLT-esterase, IFN- $\gamma$ , and TNF/LT by a protective (YA26) and a nonprotective (YA15) CTL clone. CTL were incubated with P815 target cells (-), or target cells that had been coated with 1  $\mu$ M of peptide Py 277-288 (+). Supernatants were collected after 6 h and assayed for the different secretory products. Results are expressed as the mean value of triplicate cultures.



**Figure 3.** Comparison of the association of protective (YA26) and nonprotective (YA15) CTL clones with parasitized hepatocytes. Mice were infected with sporozoites and 24 h later injected with radiolabeled CTL clones. The mice were killed 4 or 8 h after the transfer of T cells. Liver sections were observed microscopically to determine the number (per cm<sup>2</sup>) of labeled T cells (A). (B) Percentage of parasitized cells associated with YA26 or YA15 cells, obtained by counting at least 500 parasitized cells. The "association" between parasitized hepatocyte and labeled T cells is illustrated in Fig. 4. Results are expressed as the mean value  $\pm$  SE obtained by counting 5-10 sections.

terns of protective and nonprotective clones, labeled with <sup>51</sup>Cr, in the different organs of the recipient mice. We found that 2, 6, and 24 h after the adoptive transfer, cells of the protective and the nonprotective clone reached the liver, lungs, spleen, and kidneys in comparable numbers. The maximum number of T cells that reached the liver represented 24% and 17.8% of the original intravenous inoculum of YA15 and YA26 cells, respectively. These patterns of organ localization did not differ whether the adoptive transfer was performed on either naive or sporozoite-infected mice (data not shown).

To determine the precise localization of the CTL clones within the liver, we performed histological studies combined with autoradiography. <sup>35</sup>S-Methionine-labeled T cell clones

were injected into mice that had been infected 24 h before with sporozoites. Their livers were excised 4 and 8 h after the cell transfer, and the tissue sections were processed for histology and autoradiography. As shown in Fig. 3 A, there was no significant difference in the number of the two different radiolabeled T cell clones present in liver sections, confirming the previous results.

There was, however, a striking difference with regard to the relationship that parasitized hepatocytes establish with the labeled T cells of these two clones. At 4 and 8 h after adoptive transfer, labeled protective YA26 cells could be seen "associated" with 30–40% of the parasitized hepatocytes (Fig. 3 B). In contrast, in liver sections of mice that received YA15 cells, only 2–4% of parasitized cells were seen in close association with T cells (Fig. 3 B). Parasitized hepatocytes and labeled T cells were considered "associated" when found in close proximity, as illustrated in Fig. 4. Results of additional experiments carried out with a second protective CTL clone (YA23) also showed a high degree of association between parasitized hepatocytes and these T cells (data not shown).

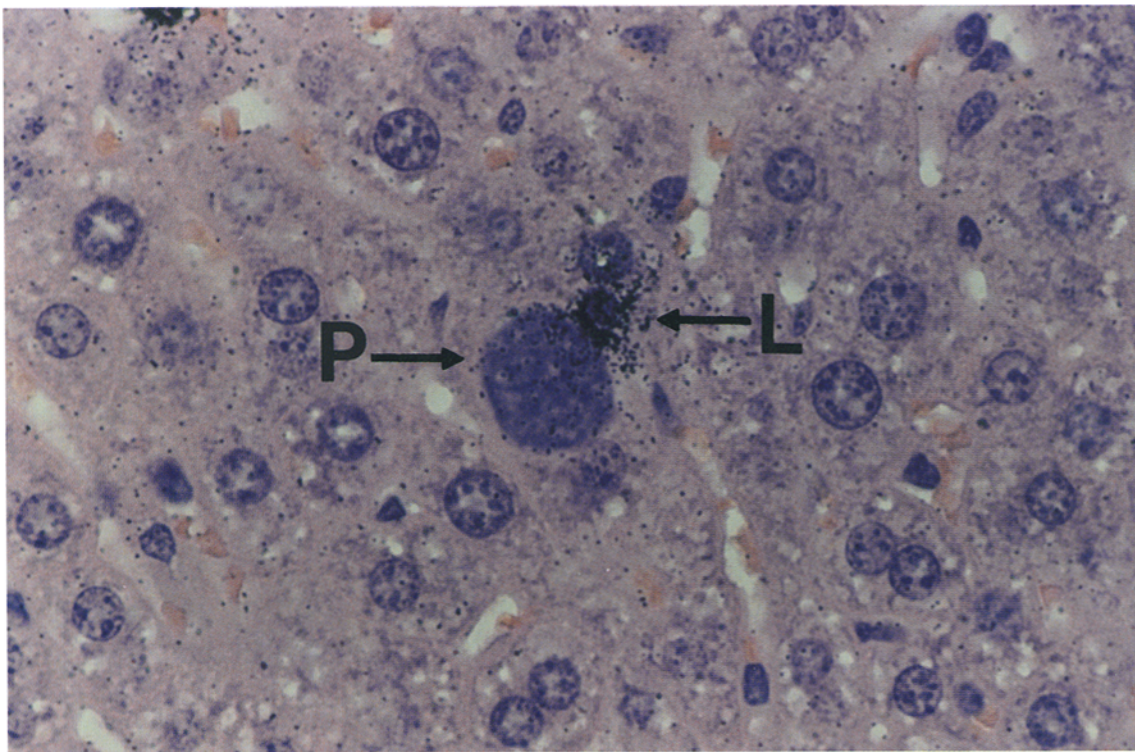
*Expression of CD44 and VLA-4 by CTL Clones Correlates with Their In Vivo Antiparasite Activity.* Our histological findings raised the possibility that protective and nonprotective clones might differ in the expression of surface molecules involved in adhesion and/or migration of T cells. To identify possible differences in the expression of such surface

molecules, FACS<sup>®</sup> analysis of YA26 and YA15 clones was performed using mAbs of various specificities.

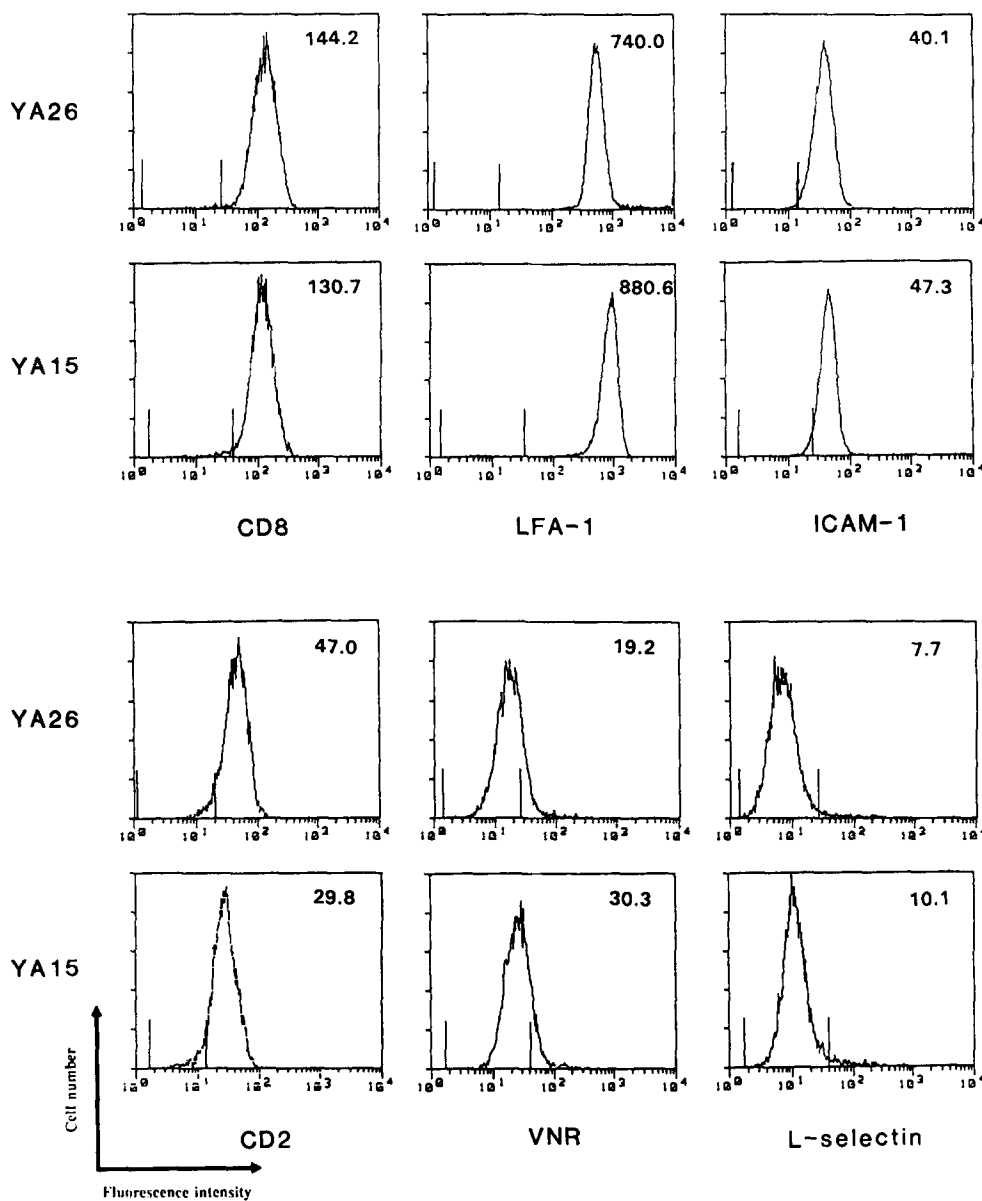
Both CTL clones display a V $\beta$  8.1 TCR, which is expressed at similar levels. No differences were found in the expression of CD45 and CD45Rb by these two T cell clones, and both were negative for CD45Ra, expressed only on naive T lymphocytes (data not shown). The analysis of surface expression of adhesion receptors revealed that while both clones failed to express L-selectin (LECAM-1/Mel-14), they expressed comparable amounts of CD8, LFA-1, ICAM-1, CD2, and vitronectin receptor (VNR) (Fig. 5).

Most revealing was the FACS<sup>®</sup> analysis with mAbs directed against CD44 and VLA-4. Comparing four different anti *P. yoelii* CTL clones, we observed a clear correlation between their protective capacity and their levels of expression of CD44 and, to a lesser degree, of VLA-4. As shown in Fig. 6, protective clones YA26 and YA23 displayed levels of CD44 expression several times higher than those of nonprotective YA15 cells, while the partially protective clone YB8 displayed intermediate levels of CD44. This pattern was observed with three different anti-CD44 mAbs (KM 703, KM 201, and IM7.8.1).

*Selection of Cells of the YB8 Clone That Express Low Levels of CD44 Generates a Nonprotective Subpopulation.* The FACS<sup>®</sup> analysis of CD44 on the surface of YB8 cells showed a broad distribution of fluorescence (Fig. 6), raising the possibility



**Figure 4.** Cells of the adoptively transferred protective CTL clone (YA26) "associate" with parasitized hepatocytes. Autoradiography of the liver section of a mouse infected with sporozoites and injected, 24 h later, with radiolabeled CTL. The mouse was killed 8 h after the adoptive cell transfer. Liver sections were processed for histology and autoradiography following standard procedures. (P) Parasitized hepatocyte; (L) labeled YA26 cell ( $\times 2,000$ ).



**Figure 5.** Expression of various T cell surface molecules in protective (YA26) and nonprotective (YA15) cells. CTL clones were stained with mAbs to various T cell surface molecules, followed by fluorescein-conjugated goat anti-rat or goat anti-hamster antibody, and analyzed by FACS®. The relative mean fluorescence obtained with each mAb is indicated on the top right corner of each histogram. The region between bars (lower left corner of each histogram) represents the background fluorescence obtained when the cells were incubated only with fluorescein conjugated second antibody.

that two subpopulations with different protective activities could be selected by sorting the cells having high and low levels of CD44. Indeed, after two rounds of sorting, we obtained two subpopulations that we designated YB8-low and YB8-high. The YB8-low subpopulation expressed levels of CD44 similar to those of the nonprotective YA15 clone. The YB8-high subpopulation expressed levels of CD44 close to those found in the original, nonsorted YB8 cells (Fig. 7 A). Both populations expressed comparable amounts of the other markers, and displayed similar levels of *in vitro* cytolytic activity. Interestingly, both subpopulations expressed identical levels of VLA-4 (data not shown).

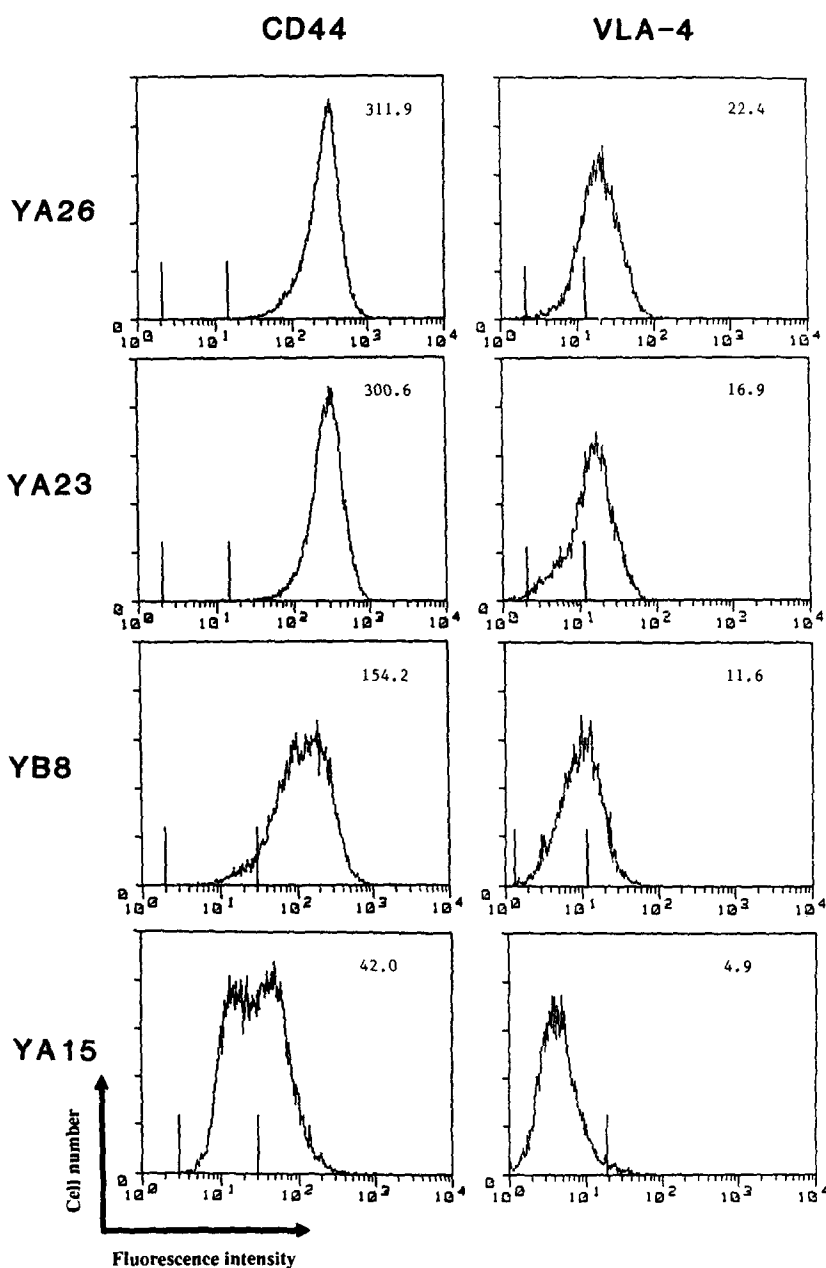
When the cells were adoptively transferred to naive BALB/c mice, the YB8-high subpopulation inhibited the development of liver stages by 61%. In contrast, the YB8-low cells had

no detectable inhibitory effect on the intrahepatocytic parasites (Fig. 7 B).

Attempts to isolate subpopulations expressing high and low levels of VLA-4, after the same procedure as for CD44, have so far been unsuccessful.

*Triggering of CD44 Fails to Promote LFA-1-mediated Adhesion of Nonprotective YA15 Cells.* Recent studies have demonstrated that the binding of anti-CD44 antibodies to the T cell surface promotes homotypic T cell adhesion mediated by LFA-1 (11). We therefore decided to determine if YA15 and YA26 clones differed in their adhesion properties, upon triggering of CD44.

Incubation of protective YA26 cells with anti-CD44 antibodies induced a strong homotypic aggregation (Fig. 8 A). This aggregation was completely inhibited by the anti-LFA-1



**Figure 6.** Protective activity of CTL clones correlates with their expression of CD44 and VLA-4. Four different antimalaria CTL clones were stained with mAbs against CD44 or VLA-4, and analyzed by FACS<sup>®</sup>. The relative mean fluorescence obtained for each clone is indicated on the top right corner of each histogram. In previous work (2), it was determined that CTL clones YA23 and YA26 eliminate parasitized cells by 90% or more, while clone YB8 eliminates 50–60% of the parasitized cells, and YA15 cells have no detectable effect.

antibody (Fig. 8 C). In contrast, anti-CD44 antibodies failed to induce aggregation of nonprotective YA15 cells (Fig. 8 B). In the presence of PMA, however, YA26, as well as YA15, cells aggregate strongly (Fig. 8, E and F, respectively). Incubation of these cells with mAbs to LFA-1 or class I MHC (H-2K<sup>d</sup>) does not induce aggregation (data not shown), in agreement with previous studies with human T cells (11).

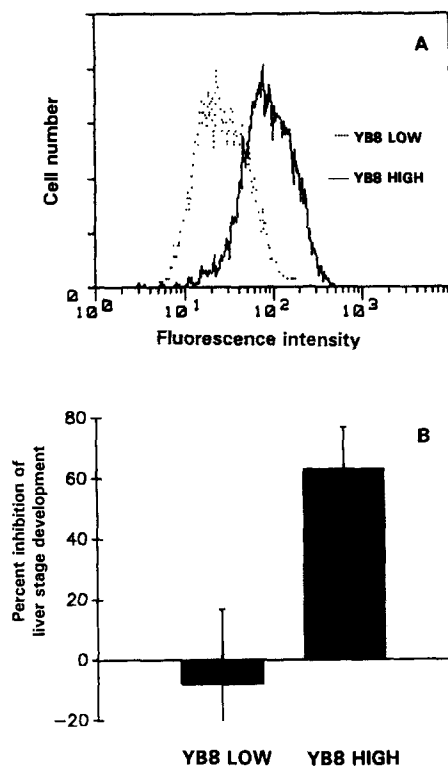
### Discussion

The basic question we have addressed in this manuscript is: What are the factors that confer to certain murine CD8<sup>+</sup> T cell clones the capacity to protect against malaria, while other CD8<sup>+</sup> clones fail to be protective?

We found that protective and nonprotective CTL clones do not differ in their fine epitope specificity and display, *in vitro*, similar levels of lysis and DNA degradation of target cells. Their pattern of production of lymphokines and granule-associated proteins does not correlate with their *in vivo* antiparasitoid activity.

The two clones, however, differed strikingly with regard to two other characteristics. First, upon adoptive transfer, we observed that T cells from the protective clone were associated with 30–40% of infected hepatocytes, while the association of the nonprotective T cell clone with infected hepatocytes was a rare event.

Second, we detected a pronounced difference in the ex-



**Figure 7.** Selection of YB8 subpopulations expressing high and low levels of CD44, generates protective and nonprotective subpopulations. (A) FACS<sup>®</sup> analysis of the two subpopulations, YB8/low (.....) and YB8/high (—), obtained by sorting the YB8 CTL clone, using a mAb to CD44. (B) The capacity of these two subpopulations to inhibit parasite development upon adoptive transfer to sporozoite infected mice.  $2.5 \times 10^7$  T cells of each subclone were adoptively transferred to naive BALB/c mice 6 h before the challenge with viable *P. yoelii* sporozoites. The percent inhibition of liver stage development was calculated in relation to the amount of parasite rRNA present in the liver of control mice, challenged with sporozoites, but not injected with T cells. Results are expressed as the mean value of four mice  $\pm$  SE.

pression of CD44 and VLA-4 in protective vs. nonprotective clones. By FACS<sup>®</sup> analysis, we found that the amount of CD44 and VLA-4 expressed in two protective clones (YA26 and YA23) was several times greater than that of the nonprotective clone YA15.

Another clone, YB8, which upon adoptive transfer induced intermediate levels of protection, i.e., a 50–60% reduction of the hepatic parasite load, displayed intermediate levels of CD44 and VLA-4. More important, we selected two subpopulations of clone YB8 expressing low and high levels of CD44. Upon adoptive transfer into infected mice, the subpopulation CD44 low failed to induce any protection, while the CD44 high subpopulation reproduced the level of inhibition seen with the original YB8 clone. It is noteworthy that both subpopulations expressed identical levels of VLA-4, supporting the notion that the protective activity of antimalaria CTL clones correlates primarily with the expression of CD44.

Lymphoid cells express a CD44 molecule with an apparent molecular mass of 85 kD, which has been implicated in sev-

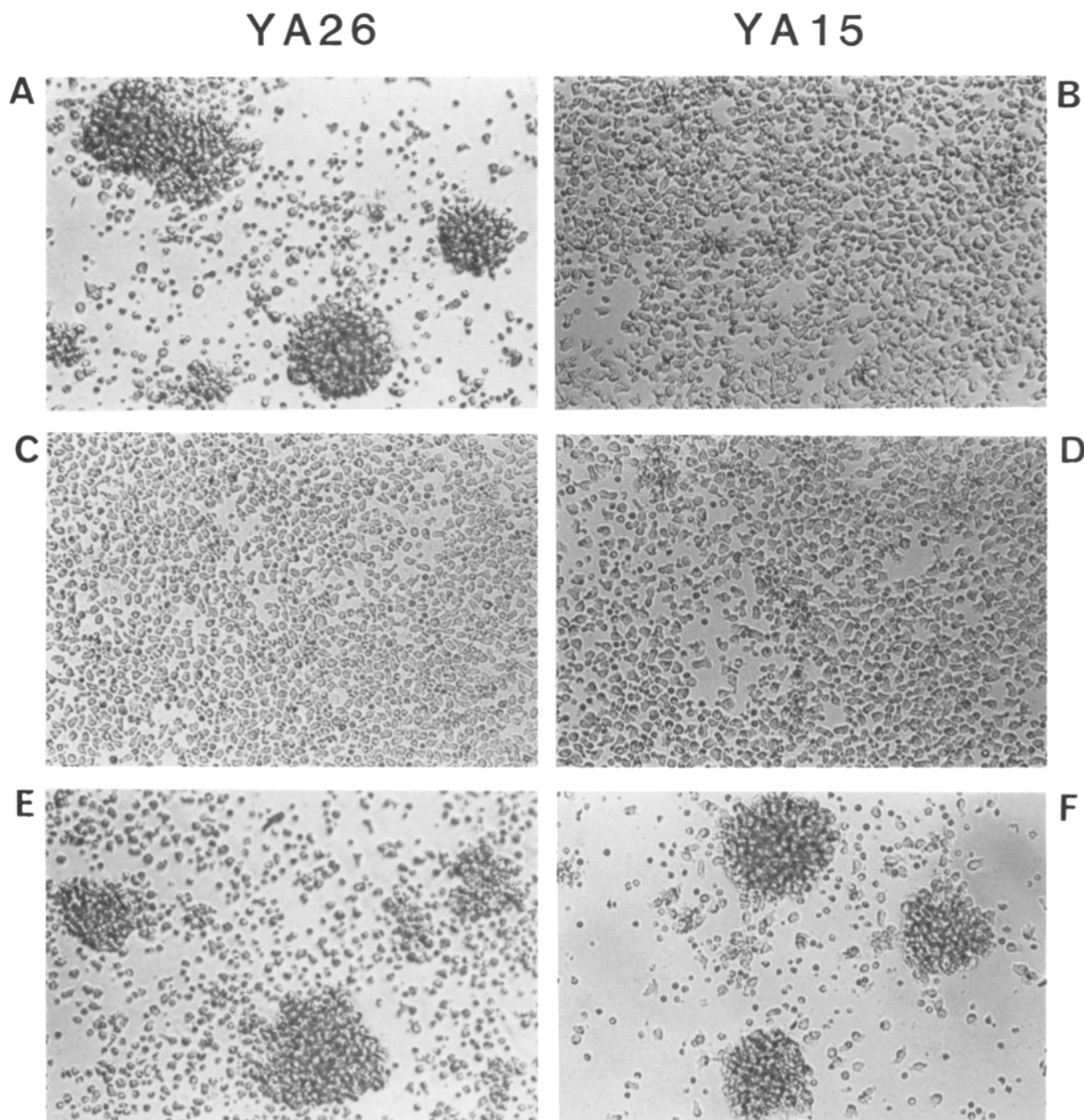
eral functions related to cell adhesion and migration (12). This molecule participates in the binding of lymphoid cells to high endothelial venules (13), to hyaluronate (14–16), and to extracellular matrix proteins (17). Also highly relevant to our observations is the fact that CD44 has been shown to mediate not only adhesion of activated T lymphocytes to human umbilical vein endothelial cells, but also their transendothelial migration (18).

The observed differences in CD44 expression by individual antimalaria CD8<sup>+</sup> CTL clones, detected by FACS<sup>®</sup> analysis, might reflect different amounts of CD44 and/or the preferential expression, by certain CTL clones, of a different isoform of this protein that is recognized less efficiently by the mAbs we used. The occurrence of distinct isoforms of CD44, which differ by the insertion of some amino acids into the extracellular domain of this protein, has recently been documented for human foreskin fibroblasts (19), the human myelomonocytic cell line KGla (20), and the rat pancreatic carcinoma Bsp73/ASML (21). It has also been found that the presence of some of these isoforms confers metastatic properties to carcinoma and lymphoma cells (21, 22). In view of these data on human and rat CD44 isoforms, it will be important to determine whether the different expression of CD44 we observed between protective and nonprotective CD8<sup>+</sup> CTL clones is only a quantitative and/or also a qualitative trait. The elucidation of this matter will require the structural characterization of these proteins and, if feasible, also the transfection of the CD44 gene from protective into nonprotective CTL clones.

The CD44 molecule has also been implicated in signal transduction, since triggering of CD44 by specific mAbs promotes LFA-1-mediated homotypic adhesion of T cells (11), and also heterotypic adhesion of B cells to follicular dendritic cells (23). It is therefore plausible that the levels of CD44 expression may determine the capacity of the antimalaria CD8<sup>+</sup> T cells to adhere to endothelial cells and migrate through intercellular spaces in the liver. In this process, the triggering of CD44 may have a crucial role in modulating LFA-1-mediated adhesiveness of the T cell clones to distinct cell types that express LFA-1 ligands (e.g., ICAM-1). This interpretation is supported by our finding that cells of the protective clone YA26 displayed strong LFA-1-mediated aggregation upon triggering of CD44, while YA15 cells were completely refractory to aggregation. Detailed *in vivo* morphological studies, using EM, and *in vitro* assays, will be required to determine whether the expression of CD44 correlates with the capacity of these CTL clones to adhere and/or migrate through endothelial and other liver cells.

Another adhesion molecule, VLA-4, was also expressed more abundantly on protective than nonprotective clones. As a member of the integrin family of adhesion molecules, VLA-4, like CD44, has been implicated in the homotypic and heterotypic adhesion of lymphocytes. This molecule is known to bind to V-CAM-1 (24), which is expressed on activated endothelial cells, and to a peptide (CS-1) of human plasma fibronectin (25). At present, we cannot discard the possibility that the higher expression of VLA-4 in the pro-





**Figure 8.** Triggering of CD44 induces LFA-1-mediated homotypic aggregation of protective YA26 cells. A protective (YA26) and a nonprotective (YA15) CTL clone were incubated with mAb to CD44 alone (A and B) or with mAb to CD44 and to LFA-1 (C and D). In E and F, each of these CTL clones was incubated with PMA. After 2 h of incubation, the cultures were photographed ( $\times 200$ ).

tective YA23 and YA26 clones contributes, together with CD44, to the ability of these clones to eliminate malaria parasites.

In conclusion, our studies have shown that antimalaria CD8<sup>+</sup> CTL clones differ in the expression of certain adhesion molecules. We found that these differences correlate closely with the ability to associate with parasitized liver cells and, most importantly, with the capacity of these CTL clones to inhibit the development and protect against intrahepatocytic stages of malaria parasites. Because nonprotective CTL clones are fully cytolytic *in vitro*, they might not be defective regarding their adhesion to target cells. It is likely that these

cells migrate inefficiently through the liver parenchyma and therefore rarely come into the vicinity of infected hepatocytes.

Previous studies have shown that resting and antigen-activated T cells differ significantly in their expression of certain adhesion molecules, including CD44. The present study shows that these differences can also be detected among antigen-activated CD8<sup>+</sup> T cells. The characterization and the perspective of modulating the regulatory mechanisms that control the expression of these adhesion molecules may, in the future, increase greatly the efficacy of certain immunological interventions, such as the therapeutic transfer of lymphokine-activated killer cells and vaccines.

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