Proc. Natl. Acad. Sci. USA
Vol. 91, pp. 1959–1963, March 1994
Immunology

Adoptive transfer of immunity to *Theileria parva* in the CD8$^+$ fraction of responding efferent lymph

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Communicated by Don W. Fawcett, September 17, 1993

ABSTRACT **Evidence that class I major histocompatibility complex-restricted cytotoxic T lymphocytes (CTL) are involved in immunity to malaria has highlighted the potential importance of these cells in protection against intracellular parasites. Parasite-specific CTL are a prominent feature of the immune response of cattle to *Theileria parva*, a related apicomplexan parasite. The relationship between the appearance of these cells in the blood of immune cattle under challenge and the clearance of infection suggests that they are involved in the control of infection, but direct evidence is lacking that CTL can mediate protection. We have made a quantitative kinetic study of CTL responses in lymph originating from infected lymph nodes in a number of immune cattle under challenge with *T. parva*. Direct killing activity and the frequency of CTL precursors (CTLp) within responding cell populations were evaluated. A substantial increase in the proportion of CD8$^+$ CTL was observed between days 8 and 11 after challenge. Frequencies of CTLp as high as 1:32 were observed and activity was essentially confined to the large blasting cell fraction. The analogous response in peripheral blood was of lower magnitude and delayed by 1–2 days. The high frequency of CTLp in efferent lymph permitted the adoptive transfer of this activity between immune and naive monozygotic twin calves. In separate experiments, naive calves lethally infected with *T. parva* were protected by inoculation of up to 10^{10} responding CD8$^+$ T cells derived from their immune twins. Elimination of CD8$^+$ T cells within the inoculum abrogated this effect. These findings provide direct evidence that CD8$^+$ T cells can control *T. parva* infections in immune cattle.**

Apicomplexan blood parasites constitute a serious threat to large sections of human and animal populations throughout the world. Probably of greatest significance in the context of Third World development are the malaria infections of man and the theilerioses of cattle. The life cycles of Plasmodium and Theileria parasites are similar in that infection occurs through inoculation of the sporozoite stage into the mammalian host by an arthropod vector, and both parasites undergo asexual schizogenous replication in nucleated cells prior to invasion of erythrocytes. However, unlike Plasmodium, Theileria parasites invade leukocytes and it is their schizogenous rather than intra-erythrocytic stage that is generally responsible for causing disease. There are also striking similarities in the immune responses elicited by the early developmental stages of these parasites. In both infections antibodies specific for sporozoite surface antigens can be generated but, although they have been shown to be protective under experimental conditions (1, 2), their presence does not correlate with protection in recovered individuals (3–5). Cytotoxic CD8$^+$ T-cell responses against parasitized hepatocytes and leukocytes have been demonstrated in infections with Plasmodium and Theileria parasites, respectively (6–8), and, in the case of Plasmodium, have been shown to confer protection in laboratory animal models (9, 10). However, there is as yet no clear evidence that these cytotoxic T-cell responses can control infections in natural human and bovine hosts.

The most important species of Theileria in Africa is *Theileria parva*, the causal agent of East Coast fever. *T. parva* infects all lineages of lymphocytes and schizogeny is associated with activation and proliferation of infected cells (11). Synchrony of division of the schizont and host cell results in rapid clonal expansion of parasitized cells. The parasite therefore remains in an intracellular location throughout much of this replicative stage of development, emerging only when merozoites are produced and infect erythrocytes. Although infection of susceptible animals results in high mortality, animals that survive or those treated with antitheilerial drugs are immune to subsequent challenge with the same strain of parasite (12). The transient appearance of parasitized cells in immune cattle undergoing challenge with *T. parva* indicates that protective immune responses in such animals are directed against infected lymphocytes (8). Cell-mediated cytotoxic activity for parasitized cells has been observed in peripheral blood during the recovery phase of primary and secondary infections. Detailed studies of the responses *in vivo* and of cytotoxic T lymphocyte (CTL) clones derived from immune cattle have demonstrated that the effectors belong to the CD8$^+$ subset of T cells, are class I major histocompatibility complex (MHC)-restricted, are specific for parasitized cells, and in some instances are parasite strain specific (8, 13).

Herein we report the results of a study designed to determine the role of CD8$^+$ T-cell responses in protection against *T. parva*. The study employed lymphatic cannulation techniques in conjunction with a limiting dilution analysis assay of CTL precursors (CTLp) to obtain precise quantitative data on the kinetics of the CTL response in relation to infection. The protective capacity of immune CD8$^+$ T cells was demonstrated by adoptive transfer of enriched populations of CD8$^+$ T cells obtained from responding lymph. The results of this study provide direct evidence that CD8$^+$ T cells can control infections with *T. parva*.

MATERIALS AND METHODS

Animals and Surgery. Boran (*Bos indicus*) cattle reared indoors under parasite-free conditions and aged between 3 and 12 months were used for the study. Monozygous twin

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Abbreviations: CTL, cytotoxic T lymphocyte(s); CTLp, CTL precursor(s); ELL, efferent lymph lymphocyte(s); mAb, monoclonal antibody; MHC, major histocompatibility complex; PBM, peripheral blood mononuclear cell(s); FACS, fluorescence-activated cell sorter.

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calves were derived from surrogate dams implanted with split embryos. Prescapular efferent lymphatic ducts were cannulated essentially as described (14).

Immunization and Challenge. Cattle were immunized with the Muguga stock of *T. parva* by inoculation with an LD_{100} of International Laboratory for Research on Animal Diseases sporozoite stabilate 3087 and simultaneous treatment with long-acting oxytetracycline (12). Lethal challenge infections were administered in the form of stabilated sporozoites or triturated salivary glands of infected *Rhipicephalus appendiculatus* ticks. Immunizing and challenge inocula were administered subcutaneously in the lateral aspect of the neck, an area drained by the prescapular lymph node (15). Progress of infection was monitored by microscopic examination of lymph node puncture biopsies after staining with fluorescein isothiocyanate-conjugated bovine antiserum to *T. parva* schizonts (8). Animals undergoing severe infections were treated with parvaquone (Clexon, Pitman–Moore).

Cell Populations. Peripheral blood mononuclear cells (PBM) were prepared by flotation on Ficoll-Paque (Pharmacia) as described (16). Efferent lymph lymphocytes (ELL) were prepared in the same manner from short-term or overnight collections of lymph. Fractionation of $CD8^+$ T cells from fresh ELL for kinetic studies was conducted on a fluorescence-activated cell sorter (FACS; Facstar Plus, Becton Dickinson) after staining with the bovine CD8-specific monoclonal antibody (mAb) ILA51 (17). Sorting on the basis of forward light scatter allowed fractionation of $CD8^+$ cells into large (blasting) and small populations. Because generation of the large numbers of cells required for *in vivo* transfer studies was not possible by cell sorting techniques, $CD8^+$ and $CD8^-$ populations were prepared from overnight collections of ELL by depletion of appropriate populations with rabbit complement and lineage-specific mAb of IgG2a isotype. Briefly, ELL for fractionation were suspended in RPMI 1640 medium (without Hepes buffer) supplemented with 10% inactivated fetal bovine serum, 2 mM L-glutamine, 50 μM 2-mercaptoethanol, and 50 μg of gentamycin per ml (complete medium) at a density of 1–5 $\times$ 10^8 cells per ml. mAbs ILA50 [bovine immunoglobulin (18)], ILA58 [bovine immunoglobulin light chain (18)], ILA12 [bovine CD4 (19)], and CC15 [WC1 specificity of bovine $\gamma\delta$ T cells (20)] were added at dilutions approximating 50 times their saturated binding concentration to delete bovine B cells, $CD4^+$ T cells, and $\gamma\delta$ T cells, respectively, the dominant non-CD8 lineages in efferent lymph (Fig. 1*A*). The cell suspensions were then incubated for 30 min on ice before the addition of rabbit serum as a source of complement. Complement killing was allowed to proceed for 40 min at 37°C and surviving cells were collected by flotation on Ficoll-Paque. The degree of enrichment was assessed by FACS analysis of surviving cells after staining with a separate panel of mAbs, all of IgG1 isotype: CC30 [bovine CD4 (21)], ILA51 [bovine CD8 (17)], ILA29 [WC1; bovine $\gamma\delta$ T cells (20)], ILA30 [bovine IgM; B cells (22)]. In some instances a second cycle of complement lysis was necessary to achieve satisfactory enrichment. To examine contaminating populations present in $CD8^+$-enriched ELL, $CD8^+$ T cells in the fraction were depleted by complement lysis with the CD8-specific mAb IL-A105 (IgG2a). Monocytes or dendritic cells were consistently absent from all ELL fractions, as determined by staining with mAb ILA24 (23).

Cell Lines. Transformed parasitized cell lines for use as targets in limiting dilution microcultures and cytotoxicity assays were prepared by *in vitro* infection of PBM with sporozoites as described (24). Uninfected control cell lines were Con A-stimulated lymphoblasts maintained by culture in the presence of 10% T-cell growth factors (25).

Assays of Cellular Cytotoxicity. Direct parasite-specific cytotoxic activity in whole and fractionated ELL and PBM was measured on ^{51}Cr-labeled *T. parva*-infected cell lines using a standard 4-hr ^{51}Cr release assay (25) conducted in flat-bottomed 96-well plates. Parasite specificity and MHC restriction of cytotoxic activity were confirmed by the inclusion in the assay of autologous Con A blasts and class I MHC-mismatched infected cell lines. The frequency of *T. parva*-specific CTLp in these populations was determined by limiting dilution analysis (26).

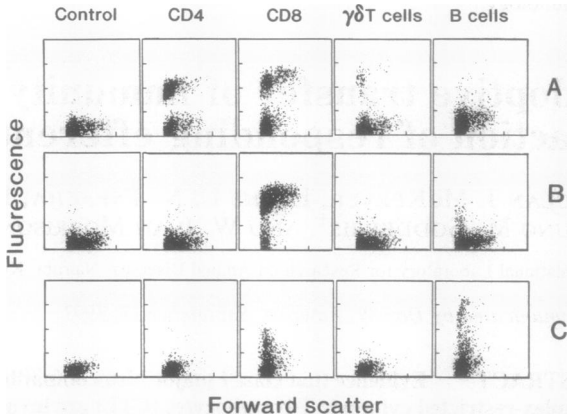

FIG. 1. Phenotypic analysis of ELL populations prepared from immune lymph collected on day 9 after challenge for cell transfer experiments. (*A*) Unfractionated ELL. (*B*) Population enriched for $CD8^+$ T cells by complement lysis of $CD4^+$ T cells, B cells, and $\gamma\delta$ T cells. (*C*) Population remaining in ELL after depletion of $CD4^+$ T cells, B cells, $\gamma\delta$ T cells, and $CD8^+$ T cells, referred to herein as the contaminant population.

Immunofluorescent Staining. Cells were stained for phenotypic analysis by flow cytometry essentially as described (27).

RESULTS

Kinetics of CTL Activity in ELL and PBM. To examine the kinetics of *T. parva*-specific CTL activity in blood and lymph of immune cattle, and to assess the intensity of these responses, a group of five calves was immunized and challenged with the parasite. Direct cytotoxic activity and CTLp frequencies were measured in ELL and PBM from these cattle at various times after challenge. In line with previous observations (8), MHC-restricted parasite-specific killing was observed in PBM and ELL from all animals and coincided roughly with the detection of schizont parasitosis in the draining lymph node. Peak cytotoxic activity was observed between days 9 and 11 and ranged from 17% to 78% at an effector-to-target ratio of 40:1. The response was first detected in efferent lymph on day 7 after challenge and appeared in peripheral blood 1 day later. Frequencies of parasite-specific CTLp in ELL and PBM were similar in samples taken prior to this response (Fig. 2*A*). In keeping with observations of direct cytotoxic activity, CTLp frequency in ELL had increased considerably by day 7 after challenge and peaked at values in excess of 1:40. As observed with direct cytotoxic activity against the parasite, this increase appeared later in PBM than ELL, and the frequencies were 20- to 30-fold less.

To locate the CTLp activity within ELL, cells were sorted to purities of 95–98% into $CD8^+$, $CD8^-$, $CD8^{+large}$, and $CD8^{+small}$ subpopulations. The frequencies of CTLp in these fractions indicated that they were all $CD8^+$ and that the large majority was blasting cells (Fig. 2*B*).

Preparation of Enriched ELL Subpopulations for Adoptive Transfer. Three sets of monozygous twin calves were identified for adoptive transfer experiments, and one individual from each set was immunized with *T. parva*. These animals

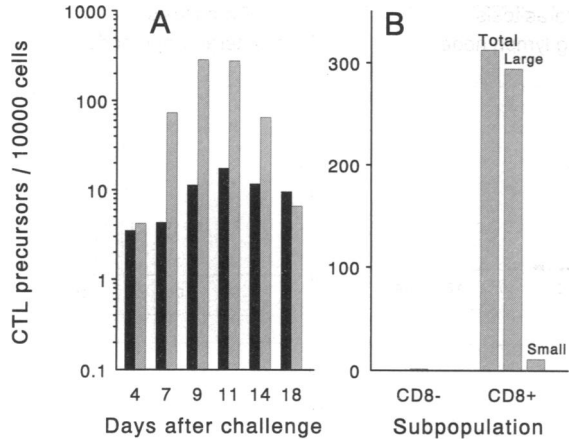

FIG. 2. (*A*) Kinetic changes in the numbers of *T. parva*-specific CTLp in PBM (■) and ELL (■) of an immune calf under challenge with the immunizing stock of the parasite. (*B*) Frequency of *T. parva*-specific CTLp in day 9 ELL fractionated on the basis of CD8 expression and cell size.

were challenged with a lethal dose of the parasite 6–8 weeks after immunization, and the efferent lymphatic of the draining lymph node was cannulated. Overnight collections of responding efferent lymph from these calves were used to prepare cell populations for adoptive transfer. $CD8^+$ T-cell-enriched fractions were prepared from two of the donors by complement lysis of non-CD8 ELL lineages. FACS analysis of these fractions revealed complete depletion of $CD4^+$ T cells and $\gamma\delta$ T cells, with small numbers of contaminating B cells remaining (Fig. 1*B*). Enrichment of CTL function was confirmed by limiting dilution analysis (Table 1). However, a contaminating population was present in these fractions that was apparently negative for the expression of CD4, CD8, and the markers used in this study to define B cells and $\gamma\delta$ T cells. It was not possible to identify a surface marker that defined this population, but subsequent phenotypic analysis after double-staining indicated that the dominant contaminating cell was $CD4^-$ $CD8^-$ $CD2^+$ $CD5^+$ $CD6^+$ (data not shown). This population could be prepared for functional analysis by complement lysis of enriched $CD8^+$ cells and was prepared in this manner from the third donor calf for adoptive transfer. When tested by limiting dilution analysis the population did not contain detectable CTLp (see Table 1) and was, in addition, incapable of killing autologous infected or uninfected cell lines at an effector-to-target ratio of 20:1 in 4-hr or overnight ^{51}Cr assays (data not shown).

Table 1. Temporal and quantitative details of three adoptive transfer experiments between immune and naive monozygous twin cattle under challenge with *T. parva*

Transfer population	Day*	No. of cells transferred	CTLp frequency in inoculum	% $CD8^+$
$CD8^+$	9 (6)	0.5×10^9	1:31	85
	10 (7)	2.0×10^9	ND	80
	11 (8)	7.5×10^9	1:67	65
	Total	1.0×10^{10}		
$CD8^+$	9 (6)	2.0×10^9	1:357	72
	10 (7)	1.5×10^9	1:300	83
	11 (8)	2.0×10^9	ND	83
	12 (9)	3.0×10^9	ND	88
	Total	8.5×10^9		
Contaminant	9 (6)	2.0×10^7	<1:20,000	
	10 (7)	1.5×10^7	<1:20,000	
	11 (8)	2.5×10^8	<1:20,000	
	12 (9)	4.5×10^8	<1:20,000	
	Total	7.4×10^8		

ND, not determined.
*Relative to challenge infection of donor; numbers in parentheses denote the day of the recipient's infection.

Adoptive Transfer of ELL Subpopulations Between Immune and Naive Monozygous Twins After Challenge with *T. parva*. In two experiments, naive calves undergoing lethal infection with *T. parva* were inoculated with enriched $CD8^+$ T cells collected during the response of their immune monozygous twins to challenge. Infections were contrived so that the peak CTL response in donor lymph would coincide with the appearance of parasites in the naive recipient, based on the kinetic observations recorded above. Donor and recipient calves were both challenged with a lethal dose of sporozoite stabilate, and in each experiment a third age-matched susceptible control calf was also infected. The overnight harvests of lymph collected on days 9, 10, 11, and 12 after challenge were used as sources of $CD8^+$ cells for adoptive transfer. This period corresponded to days 6, 7, 8, and 9 of the recipient animal's infection. On each day of transfer, $CD8^+$-enriched ELL were suspended in 20 ml of autologous plasma or fetal bovine serum and administered intravenously to the recipient. Quantitative details of these transfer experiments are summarized in Table 1. In both experiments the viability and effector function of transferred CTL in the recipient animals were confirmed by their detection in PBM after transfer. In one of these animals CTL were detected in PBM at a frequency of 1:7443 on the third day of transfer, by which time it had received 2.5×10^9 cells (Table 1). Direct cytotoxic activity was detected in PBM a day later and this reached maximum levels 4 days after the final transfer (data not shown).

The febrile reactions and kinetics of schizont parasitosis observed in recipient and challenge control calves of these experiments are illustrated in Fig. 3. Schizont-infected cells were detected in the aspirates collected from the draining lymph nodes of all animals between days 6 and 7 after challenge. In the control calves the degree of parasitosis increased steadily and by day 10 of infection had spread to other peripheral lymph nodes. All of the control animals developed clinical disease and required treatment on day 14, with 30–50% parasitosis in the draining node and up to 20% of the cells of the contralateral lymph node being infected. In contrast, challenge infections in both recipients of $CD8^+$-enriched ELL were cleared without treatment. Parasites were first detected on day 7 in these animals. In the first of these experiments (Fig. 3 *Top*), parasitosis peaked at only 2% on day 9 after challenge and was undetectable by day 12. In the second experiment (Fig. 3 *Middle*), although the proportion of parasitized cells in the draining node reached 12% on day 10, the progress of infection was thereafter controlled. Parasites were not detected in the contralateral lymph node of either of these animals at any stage after challenge.

To ensure that the protection conferred on the recipient calves was not the result of the transfer of $CD8^-$ lineages contaminating the enriched $CD8^+$ fraction, a third experiment was conducted that involved the transfer of this population. The proportion of B cells remaining after complement lysis was somewhat higher than that seen in previous experiments (Fig. 1*C*) and this was possibly attributable to allelic variation in the immunoglobulin determinants recognized by ILA50 and ILA58. In addition, a small proportion of cells showing weak expression of CD8 consistently evaded lysis. A total of 7.35×10^8 cells of the contaminating fraction was transferred from the immune to the naive twin over a 4-day period. The kinetics of CTL activity in the donor twin was similar to that seen in other immune animals, and the timing of cell transfer was identical to that of the two previous experiments. However, severe clinical reactions were ob-

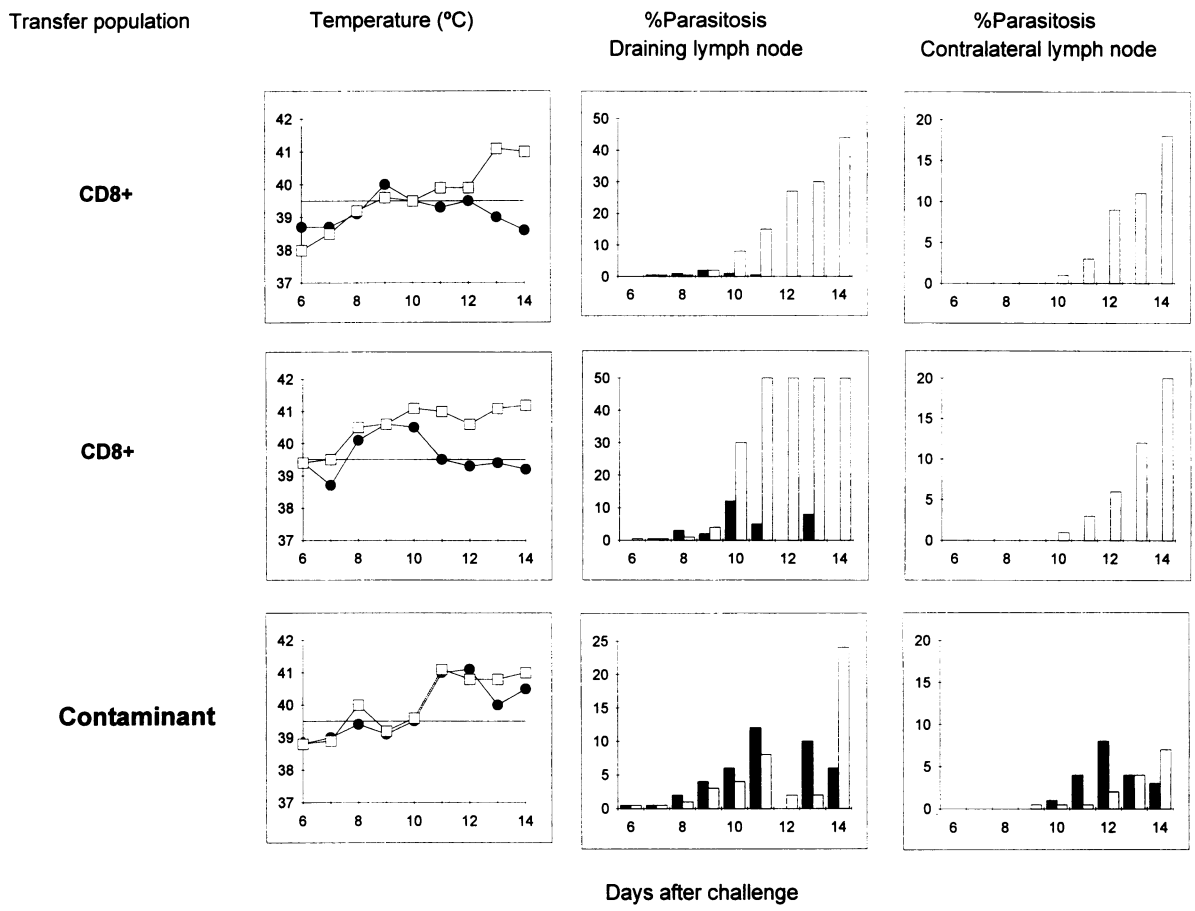

FIG. 3. Clinical and parasitological features of *T. parva* infections in untreated control calves and recipient calves after adoptive transfer of immune $CD8^+$ T cells (*Top* and *Middle*) and $CD8^-$ cells that persist after complement lysis of efferent lymph lymphocytes expressing CD4, CD8, surface immunoglobulin, and the WC1 bovine γδ T-cell marker (*Bottom*). Temperature reactions in control (□) and recipient (●) calves and parasitosis in lymph nodes of control (□) and recipient (■) calves are indicated; >39.5°C was considered febrile. Control calves of each experiment and the recipient animal of the contaminant $CD8^-$ population were treated on day 14 of infection.

served in recipient and control animals that necessitated their treatment on day 14 of infection (Fig. 3 *Bottom*).

DISCUSSION

The results of this study provide direct evidence that $CD8^+$ T cells can protect immune cattle against challenge with *T. parva*. In two separate experiments, lethally infected non-immune calves received inoculations of enriched $CD8^+$ responding T cells derived from their immune monozygous twins to coincide with the emergence of patent schizont parasitosis. In both animals the challenge infections were subsequently confined to the regional lymph node and cleared. Challenge control calves in each experiment developed generalized infections that required treatment. Several features of the study support the conclusion that this effect is mediated by parasite-specific class I MHC-restricted CTL. In line with previous observations, the clearance of challenge infections by immune cattle was in these experiments kinetically associated with the appearance of parasite-specific CTL in the circulation. Quantitative analysis of CTL activity in responding ELL after challenge with the parasite confirmed this association and, in addition, illustrated the vigor of the nodal CTL response, with frequencies of parasite-specific CTL as high as 1:31 at the peak of the response (Fig. 2*A*).

Previous studies of the kinetics of *T. parva*-specific CTL responses in immune cattle have shown that activity appears on day 8 after challenge, reaches peak levels on day 9 or 10, and thereafter disappears rapidly (8). These observations were based on assays of direct killing activity in PBM and are difficult to interpret in quantitative terms. Limiting dilution analysis has been shown to provide a more sensitive measure of *T. parva*-specific CTL activity in PBM and also allows an assessment of its intensity (26). However, because immune responses are generated in the lymph node that drains the site of antigenic challenge (28), the kinetics and intensity of *T. parva*-specific CTL responses can be analyzed more directly in cell populations leaving the responding node. CTL activity was present in ELL 1 day earlier than in PBM and at this time a substantial increase in the numbers of CTLp in lymph was already apparent. At the peak of the nodal response to infection, ELL contained parasite-specific CTLp at frequencies as much as 25 times those observed in PBM. The restriction of the CTL activity to the blasting cells of efferent lymph indicates that this response was generated within the node. Assuming a 1- to 2-day period for the expansion of sufficient numbers of parasitized cells, their kinetics are consistent with 4–5 days being required for the generation of *T. parva*-specific CTL responses in immune cattle.

In vivo cell transfer experiments with cloned and polyclonal cell lines have been used in other species to validate the effector function of cell populations with defined specificities (9, 29–32). Although *T. parva*-specific CTL lines can be generated in the laboratory with relative ease, such experiments are not practical in cattle because of the difficulties associated with generating sufficient numbers of cultured cells. The large quantities of *T. parva*-specific CTLp in responding lymph represented an ideal alternative source of CTL whose specificities were clearly relevant to *in vivo*

responses. Although complement lysis of $CD8^-$ lineages normally present in lymph provided a highly enriched $CD8^+$ fraction, a contaminating $CD8^-$ population consistently remained that was not apparent in unfractionated ELL. The majority of these cells were found to be $CD4^-$ $CD8^-$ $CD2^+$ $CD5^+$ $CD6^+$. This population contained no parasite-specific CTLp (Table 1) or nonspecific cytolytic activity for MHC-mismatched infected cells or uninfected autologous blasts (data not shown). In addition, when transferred between immune and naive twins in a third experiment, these cells did not confer protection. The function of this population remains unclear, but its phenotype suggests that it may be analogous to the double-negative $\alpha\beta$ T-cell receptor expressing T cells described in peripheral tissues of mouse and man (reviewed in ref. 33). As evidenced by limiting dilution analysis, the major *T. parva*-specific activity associated with the $CD8^+$ T-cell fraction of responding lymph was class I MHC-restricted CTL function. Although the presence of alternative mechanisms in this population cannot be ruled out, it is therefore likely that the protection conferred by transfer of enriched $CD8^+$ ELL in these experiments was mediated by parasite-specific CTL. It is improbable that the control of infection in the recipient calves was due to endogenous CTL responses. It has been reported previously that parasite-specific CTL responses cannot be detected in naive cattle undergoing lethal challenge with *T. parva* (34) and the control animals in our experiments did not develop detectable CTL activity or CTLp at any time during the course of infection (data not shown). The argument that endogenous responses might somehow have been assisted by elements of the transfer inoculum is disputed by the rapid appearance of CTLp in the blood of the recipients after transfer. In one calf, CTL activity was detected after only two daily inoculations, by which time it had received 2.5×10^9 enriched $CD8^+$ lymphocytes (Table 1). When compared with those observed for the generation of CTL responses in immune cattle, these kinetics are not consistent with endogenous CTL activity.

Although the results of this study indicate that immune $CD4^+$ lymphocytes are not required for the control of *T. parva* infections in cattle, our experiments did not determine whether these cells are capable of clearing the parasite. In addition, their role in the induction of parasite-specific CTL remains unclear. Experiments in other systems have provided equivocal data on the relative roles of $CD8^+$ and $CD4^+$ T cells in the resolution of infections with intracellular pathogens. Both populations are capable of clearing influenza infections of mice (29–31), and similar observations have been made in murine malaria (9, 32). It is therefore possible that *T. parva*-immune $CD4^+$ cells are capable of effector functions that might be significant in the absence of $CD8^+$ CTL immunity. Additional transfer experiments are necessary to examine these possibilities.

We are grateful to Drs. Torbin Jordt and David Kennedy for the generation of monozygous twin calves. This is International Laboratory for Research on Animal Diseases publication no. 1064.

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