INFECTION AND IMMUNITY, Nov. 1990, p. 3574–3581
0019-9567/90/113574-08$02.00/0
Copyright © 1990, American Society for Microbiology
Vol. 58, No. 11

Immunization with *Theileria parva* Parasites from Buffaloes Results in Generation of Cytotoxic T Cells Which Recognize Antigens Common among Cells Infected with Stocks of *T. parva parva*, *T. parva bovis*, and *T. parva lawrencei*†

T. M. KARIUKI, J. G. GROOTENHUIS,‡ T. T. DOLAN, R. P. BISHOP, AND C. L. BALDWIN§*

International Laboratory for Research on Animal Diseases, P.O. Box 30709, Nairobi, Kenya

Received 5 June 1990/Accepted 10 August 1990

Immunity to infection by the protozoan parasite *Theileria parva* in cattle is partially attributable to cytotoxic T cells, which kill lymphocytes infected with the schizont stage of the parasite. Here we evaluated five stocks of buffalo-derived *T. parva lawrencei* parasites and two stocks of cattle-derived *T. parva parva* parasites for their ability to induce in vivo cytotoxic T cells which can kill lymphocytes infected with a wide variety of strains of *T. parva* parasites. A group of seven full-sibling cattle, produced by embryo transfer and matched for at least one major histocompatibility complex class I haplotype, were immunized by infection and treatment with the parasite stocks. Target cells used in in vitro cytotoxicity assays were infected with five buffalo-derived parasite stocks and five cattle-derived parasite stocks, including *T. parva parva* and *T. parva bovis*. Immunization with any of the seven parasite stocks resulted in the generation of cytotoxic T cells which recognized parasite antigens on most if not all of the target cell lines tested, although the *T. parva bovis* stock was the least effective at doing so. Further in-depth analyses performed with peripheral blood mononuclear cells from one of the cattle immunized with *T. parva lawrencei* parasites showed that the pattern of killing of the panel of target cells was altered when either cells infected with different parasite stocks or clones of infected cells were used as stimulator cells in vitro, suggesting the presence of more than one population of parasite-specific cytotoxic effector cells in the peripheral blood mononuclear cells. However, clones of these cytotoxic effector cells recognized common or cross-reactive antigen epitopes expressed by the entire panel of infected target cells. These T-cell clones will be useful for identifying common T-cell antigen epitopes of *T. parva* and the parasite genes encoding them.

The protozoan parasite *Theileria parva* is the causative agent of the tick-transmitted cattle disease East Coast fever. East Coast fever is endemic in a large area of East and Central Africa, where it is an important cause of mortality in cattle. The existence of multiple strains of the parasite is a major hindrance to exploitation of the currently available method of immunization by infection and treatment, since immunization with individual parasite stocks may not protect against challenge with all heterologous stocks (8, 17, 27–29). However, animals immunized with buffalo-derived *T. parva lawrencei* parasites are frequently protected against a broader spectrum of parasite stocks, including both *T. parva lawrencei* and *T. parva parva* (11, 29, 31).

Immunity is at least partially attributable to a cell-mediated immune response which includes cytotoxic T cells, which kill parasite-infected cells (12, 15, 24, 26). It is believed that immune T cells recognize parasite-derived antigens on the surfaces of infected cells in conjunction with self major histocompatibility complex (MHC) antigens. The differences in the abilities of different parasite strains to induce cross-protective responses are probably a result of antigenic differences in these cell surface determinants. Both bovine helper (2) and cytotoxic (16) T-cell clones which recognize strain-specific antigens on the surfaces of infected cells have been generated in vitro, as have cytotoxic T-cell clones which recognize antigens common to cells infected with *T. parva parva* (Muguga) and *T. parva parva* (Marikebuni) (23). Cytotoxic T cells from naturally resistant African buffaloes have also been described (3), and it has been hypothesized that they recognize parasite-derived antigens which are common among cells infected with different parasite strains (3), thus providing immunity to a wide range of parasite strains. Preferential induction of cytotoxic cells recognizing common parasite antigens could be the result of a prevalence of these antigens on cells infected with stocks of buffalo-derived *T. parva lawrencei* parasites.

In this study, we evaluated the parasite strain specificities of cytotoxic T cells generated in cattle immunized with one of five different stocks of buffalo-derived *T. parva lawrencei* parasites and compared them with those generated in cattle immunized with the cattle-derived parasite stock *T. parva parva* (Uganda) or *T. parva bovis* (Boleni). These cattle-derived stocks were chosen to compare with the buffalo-derived stocks because they have been reported to provide protection against infection with a large variety of other parasite stocks (17a). T cells recognizing parasite antigens common among cells infected with different *T. parva* parasite strains would be useful in further studies for identifying parasite-derived peptides which contain common antigen epitopes and the genes coding for these peptides, with a view

* Corresponding author.
† Publication no. 769 of the International Laboratory for Research on Animal Diseases.
‡ Present address: National Veterinary Research Centre, Kenya Agricultural Research Institute, Kabete, Kenya.
§ Present address: Department of Microbiology, College of Biological Sciences, The Ohio State University, 484 West 12th Ave., Columbus, OH 43210.

towards constructing a subunit recombinant vaccine against East Coast fever.

MATERIALS AND METHODS

Parasite stocks and infection of ticks. *Rhipicephalus appendiculatus* ticks, maintained as a colony at the International Laboratory for Research on Animal Diseases (ILRAD), were infected by allowing them to feed on infected buffaloes or cattle, and sporozoite stabilates were made from them as described previously (9, 19). Infected buffaloes used for parasite stock isolations were numbers 7013, 7014, and 7065 captured in the Nanyuki area of Kenya and numbers 6998 and 6999 from the Mara area of Kenya. They were maintained in captivity at the Veterinary Research Laboratories, Kabete, Kenya. The *T. parva* parasite stocks derived from the buffaloes are referred to as *T. parva lawrencei* and designated with the appropriate buffalo number. The cattle-derived stocks used were *T. parva bovis* (Boleni), ILRAD 3039A (21); *T. parva parva* (Muguga), ILRAD 3087 (4); *T. parva parva* (Mariakani), ILRAD 3029 (17); *T. parva parva* (Marikebuni), ILRAD 3014 (17); and *T. parva parva* (Uganda), ILRAD 3066A.

Immunization of cattle. Seven Boran (*Bos indicus*) female or castrated male cattle of the same age were used for immunization. They were the progeny of a single bull and cow, produced by embryo transfer (18), and had BoLA class I phenotypes w2/w6 and KN8/KN12, respectively. The cattle identified as D487, D503, and D504 had the serologically defined BoLA class I phenotype w6/KN12, and the cattle identified as D482, D494, D505, and D508 had the phenotype w6/KN8. All seven cattle were negative for antibodies to *Theileria*, *Babesia*, *Trypanosoma*, and *Anaplasma* species and were clinically normal at the outset of the study. They were immunized with *T. parva* parasites by the method of infection and treatment (28) with frozen material as follows: D482 with *T. parva lawrencei* (7014), D487 with *T. parva bovis* (Boleni), D494 with *T. parva lawrencei* (6999), D503 with *T. parva parva* (Uganda), D504 with *T. parva lawrencei* (6998), D505 with *T. parva lawrencei* (7013), and D508 with *T. parva lawrencei* (7065). All the animals except *T. parva bovis* (Boleni)-infected animal D487 were treated after 4 days with a second dose of long-acting oxytetracycline (Terramycin LA; Pfizer, Sandwich, United Kingdom), and some showing severe reactions were treated with parvaquone (Clexon; Wellcome, Nairobi, Kenya) (10).

Isolation of PBM. Blood was collected from the jugular veins of cattle into Alsever anticoagulant solution, and peripheral blood mononuclear cells (PBM) were separated by Ficoll-Hypaque centrifugation (20).

***T. parva*-infected cell lines.** Before they were immunized, two of the seven experimental cattle, D482 and D504, served as donors of PBM for in vitro infection with *T. parva* sporozoites to establish the infected cell lines (5) used as target and stimulator cells. Infected cell lines are designated by the animal number from which the lymphocytes were obtained, followed by the parasite stock with which the lymphocytes were infected, e.g., D482 *T. parva parva* (Muguga). Seven days after infection, PBM from animal D482, which was infected in vitro with sporozoites of *T. parva lawrencei* (7014), were cloned by limiting dilution as described previously (3).

To compare the antigenic compositions of the various parasite stocks, we tested cell lines infected with buffalo- or cattle-derived parasite stocks by the immunofluorescent-antibody test (13) with monoclonal antibodies (MAb) to *Theileria* schizont antigens (22, 25). Restriction fragment length polymorphisms among the cell lines infected with buffalo-derived parasites were examined with a *T. parva*-specific repetitive DNA probe (1, 6).

Uninfected lymphoblasts for use as control target cells in cytotoxicity assays were established and maintained as described previously (3).

MHC typing of infected cell lines. PBM and parasitized cell lines were typed for their MHC class I antigens as described previously (30).

Generation and assay of cytotoxic cells in bulk cultures. To generate cytotoxic cells, we stimulated PBM in vitro in the autologous *Theileria* mixed-leukocyte reaction with an optimal number of stimulator cells essentially as described previously (14, 15) and tested them for cytotoxic activity in a 4-h ^{111}In release assay (16).

Cloning of effectors. Bulk-cultured PBM which had been stimulated three times in vitro with gamma-irradiated *T. parva*-infected cells were depleted of BoT4$^+$ cells and cloned as described previously (16). After 2 weeks of culturing, wells were screened for cell growth, and cells were tested for cytotoxic activity as indicated above and expanded. To expand the cloned cells, we distributed the contents of an individual well into 96 wells of round-bottom microtiter plates. A cell suspension (100 μl) containing 5×10^4 autologous irradiated infected cells and 30% T-cell growth factor in RPMI 1640 (without *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES]) with 10% heat-inactivated fetal bovine serum, 50 μg of gentamicin per ml, and 5×10^{-5} M 2-mercaptoethanol (complete culture medium) was added to each well, and the plates were incubated at 38°C in a humidified incubator containing 5% CO_2 in air. After 2 to 3 weeks, the cloned cells were further expanded in 96-well round-bottom microtiter plates as described above or in 24-well culture plates by seeding 2×10^5 cloned cells and 1×10^6 to 2×10^6 irradiated infected cells per well in a total volume of 2 ml of complete culture medium containing 15% T-cell growth factor.

RESULTS

Characterization of parasite stocks. To compare the parasite stocks used here for differences or apparent identity, we stained the infected cell lines with MAb to schizont membrane antigens (Table 1). In some cases, a different schizont antigen profile was obtained when cells from different animals were infected with the same parasite stocks. For example, cell lines D482 *T. parva lawrencei* (7014) and D504 *T. parva lawrencei* (7014) differed in their reactivities with MAb 15 and 18, and cell lines D482 *T. parva lawrencei* (7013) and D504 *T. parva lawrencei* (7013) differed in their reactivities with MAb 15. In other instances, cells infected with different parasite stocks had identical antigen profiles: D482 cells infected with the parasite stock *T. parva lawrencei* (7013) had the same profile as D482 cells infected with *T. parva lawrencei* (6999), and D504 *T. parva lawrencei* (7013) had the same profile as D482 *T. parva lawrencei* (7014). Among the cell lines infected with the cattle-derived parasite stocks, the schizont antigen profiles were the same for PBM from different cattle infected with the same parasite stock, except for those infected with *T. parva parva* (Uganda), which differed in their reactivities with MAb 18.

Probing of DNA from the 10 cell lines infected with buffalo-derived parasites with a 623-bp repetitive DNA fragment cloned from the genome of *T. parva parva* (Muguga) (1) revealed parasite heterogeneity in addition to that demon-

TABLE 1. MAb profiles of schizont antigens analyzed by the immunofluorescent-antibody test

T. parva-infected cell line	Reactivity of MAb:																			
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
D482 *T. parva lawrencei* (7014)	+	−	−	+	+	+	+	+	+	+	+	+	+	+	+	−	−	+	−	+
D504 *T. parva lawrencei* (7014)	+	−	−	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−	−	+
D482 *T. parva lawrencei* (7013)	+	−	−	+	+	+	+	+	+	+	+	+	+	+	−	−	−	+	−	+
D504 *T. parva lawrencei* (7013)	+	−	−	+	+	+	−	+	+	+	+	+	+	+	+	−	−	+	−	+
D482 *T. parva lawrencei* (6998)	+	−	−	+	+	+	+	+	+	+	+	+	+	−	−	−	−	+	−	+
D504 *T. parva lawrencei* (6998)	+	−	−	+	+	+	+	+	+	+	+	+	+	−	−	−	−	+	−	+
D482 *T. parva lawrencei* (6999)	+	−	−	+	+	+	+	+	+	+	+	+	+	+	−	−	−	+	−	+
D504 *T. parva lawrencei* (6999)	+	−	−	+	+	+	+	+	+	+	+	+	+	+	−	−	−	+	−	+
D482 *T. parva lawrencei* (7065)	+	−	−	+	+	+	+	+	+	+	+	+	+	−	−	−	−	−	−	+
D504 *T. parva lawrencei* (7065)	+	−	−	+	+	+	+	+	+	+	+	+	+	−	−	−	−	−	−	+
D482 *T. parva parva* (Muguga)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−	−	−
D504 *T. parva parva* (Muguga)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−	−	−
D482 *T. parva parva* (Uganda)	+	−	−	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−	−	−
D504 *T. parva parva* (Uganda)	+	−	−	+	+	+	+	+	+	+	+	+	+	+	−	−	−	+	−	−
D482 *T. parva parva* (Marikebuni)	+	−	−	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−
D504 *T. parva parva* (Marikebuni)	+	−	−	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−
D482 *T. parva parva* (Mariakani)	+	−	−	+	+	+	+	+	+	+	+	+	+	+	+	−	−	+	−	−
D504 *T. parva parva* (Mariakani)	+	−	−	+	+	+	+	+	+	+	+	+	+	+	+	−	−	+	−	−
D482 *T. parva bovis* (Boleni)	+	−	−	+	+	+	−	+	+	+	+	+	+	+	+	−	−	+	−	−
D504 *T. parva bovis* (Boleni)	+	−	−	+	+	+	−	+	+	+	+	+	+	+	+	−	−	+	−	−

strated by the MAb profiles. All cell lines exhibited distinct restriction fragment length polymorphism profiles, except for D482 *T. parva lawrencei* (6998) and D504 *T. parva lawrencei* (6998), which showed no obvious differences (Fig. 1). When identical quantities of DNA from the infected cells were analyzed, three cell lines, D504 *T. parva lawrencei* (7065), D504 *T. parva lawrencei* (7014), and D482 *T. parva lawrencei* (7065), produced stronger signals than did the others, suggesting either that they had a greater degree of homology with the probe or that there was a greater proportion of parasite DNA to host cell DNA in these infected cell lines. Some cell lines which had identical MAb profiles, e.g., D482 *T. parva lawrencei* (7013) and D482 *T. parva lawrencei* (6999), were markedly different when assessed for restriction fragment length polymorphisms with the repetitive-sequence DNA probe (Fig. 1, lanes 8 and 10).

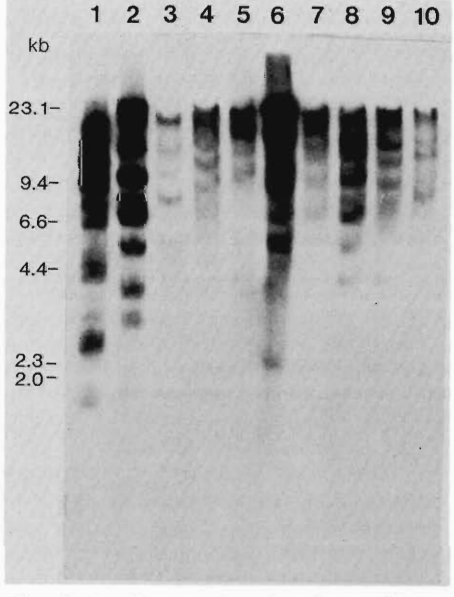

FIG. 1. Restriction fragment length polymorphism analysis of cell lines infected with parasite stocks derived from buffaloes. *Eco*RI-restricted DNAs from D504 cells infected with *T. parva lawrencei* (7065) (lane 1), (7014) (lane 2), (7013) (lane 3), (6998) (lane 4), and (6999) (lane 5) and from D482 cells infected with *T. parva lawrencei* (7065) (lane 6), (7014) (lane 7), (7013) (lane 8), (6998) (lane 9), and (6999) (lane 10) were transferred onto nitrocellulose and probed with a radiolabeled 623-bp repetitive DNA fragment from *T. parva parva* (Muguga). Filters were washed with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at 65°C. The size markers were lambda DNAs digested with *Hin*dIII; sizes are given in kilobases.

Cytotoxic response of bulk cultures from immune animals. To assess the parasite strain specificity of PBM from the immunized cattle, we tested them for cytotoxicity on the panel of target cells. PBM were stimulated two, three, or four times in vitro with cells infected with the same parasite stock as that which was used to immunize the animal. In most instances, maximal cytotoxicity for the infected target cells occurred after two or three stimulations (Fig. 2). No killing of uninfected blast cells ever occurred.

A summary of the maximal cytotoxic responses is presented in Fig. 3. Cytotoxic cells from D482 [immunized with *T. parva lawrencei* (7014)] yielded more than 50% killing of all the target cell lines infected with buffalo-derived stocks and those infected with *T. parva parva* (Uganda), *T. parva parva* (Marikebuni), and *T. parva bovis* (Boleni), about 45% killing of *T. parva parva* (Mariakani)-infected cells, and virtually no killing of *T. parva parva* (Muguga)-infected cells, even after four stimulations of the PBM in vitro (Fig. 2). A similar pattern of killing was seen with cytotoxic cells from D505 [immunized with *T. parva lawrencei* (7013)] and D508 [immunized with *T. parva lawrencei* (7065)]. Cytotoxic cells from D504 [immunized with *T. parva lawrencei* (6998)] differed in that they did not kill target cells infected with *T. parva lawrencei* (6999), were less than 50% cytotoxic for *T. parva lawrencei* (7014)-infected cells, and killed comparatively more *T. parva parva* (Muguga)-infected cells. Cytotoxic cells from D494 [immunized with *T. parva lawrencei* (6999)] yielded high levels of killing of target cells infected with *T. parva parva* (Marikebuni), *T. parva lawrencei* (7014),

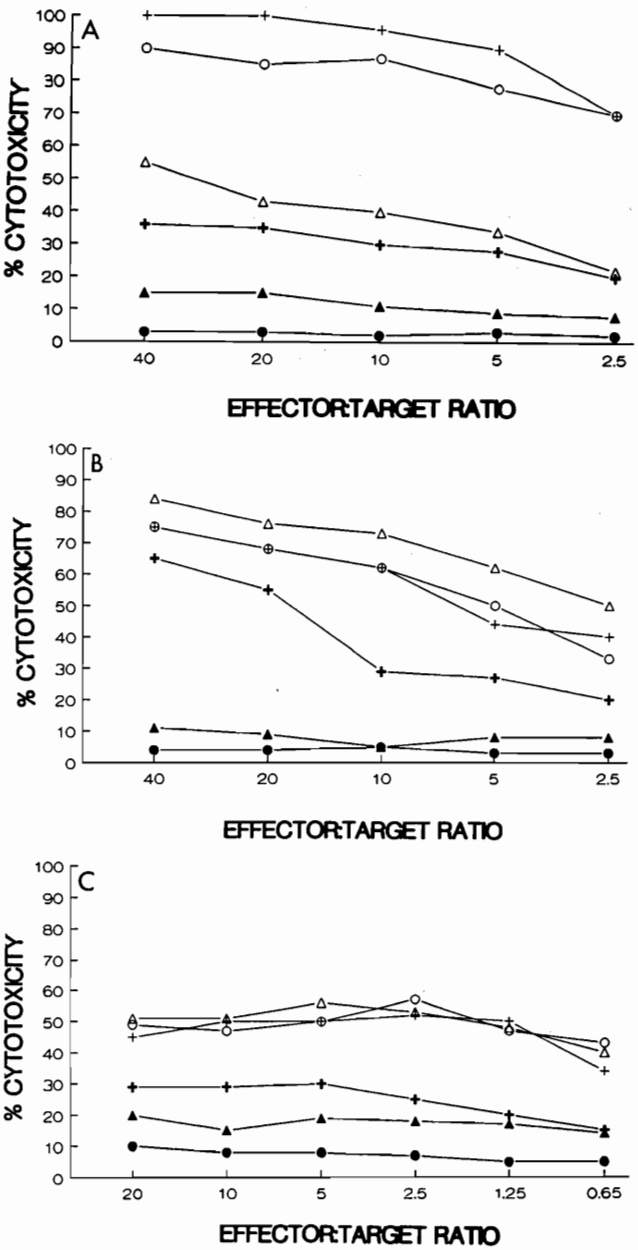

FIG. 2. Analysis of the parasite strain specificities of cytotoxic cells from animal D482 stimulated with cells infected with *T. parva lawrencei* (7014) and tested on target cells infected with the following buffalo- or cattle-derived *T. parva* parasites: +, *T. parva lawrencei* (7014); △, *T. parva lawrencei* (7013); ○, *T. parva parva* (Marikebuni); +, *T. parva parva* (Uganda); ▲, *T. parva parva* (Muguga); and ●, none (uninfected blast cells). They were tested after two (A), three (B), or four (C) stimulations in vitro.

and *T. parva lawrencei* (6998), intermediate levels of killing of those infected with *T. parva lawrencei* (7065) and *T. parva lawrencei* (6999), and low levels of killing of those infected with *T. parva lawrencei* (7013), *T. parva parva* (Uganda), *T. parva parva* (Muguga), *T. parva parva* (Mariakani), and *T. parva bovis* (Boleni).

Cytotoxic cells from the steer immunized with *T. parva parva* (Uganda), D503, yielded more than 50% cytotoxicity for all the infected target cell lines, except for those infected with *T. parva lawrencei* (6998) and *T. parva lawrencei*

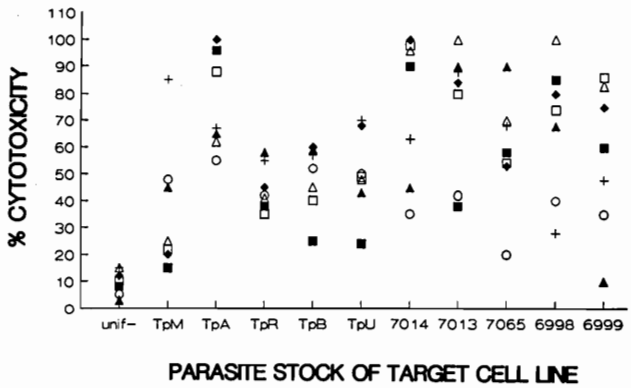

FIG. 3. Maximal cytotoxic responses of PBM from the following seven immune cattle after two, three, or four stimulations in vitro at an effector/target ratio of 40:1 or lower: ▲, D504 stimulated with *T. parva lawrencei* (6998); +, D503 stimulated with *T. parva parva* (Uganda); ○, D487 stimulated with *T. parva bovis* (Boleni); ◆, D482 stimulated with *T. parva lawrencei* (7014); ■, D494 stimulated with *T. parva lawrencei* (6999); □, D505 stimulated with *T. parva lawrencei* (7013); and △, D508 stimulated with *T. parva lawrencei* (7065). Target cell lines: TpM, *T. parva parva* (Muguga); TpA, *T. parva parva* (Marikebuni); TpR, *T. parva parva* (Mariakani); TpB, *T. parva bovis* (Boleni); TpU, *T. parva parva* (Uganda); 6998, 6999, 7013, 7014, and 7065 are *T. parva lawrencei* parasites from the buffalo number indicated; unif−, uninfected blast cells.

(6999). The most marked feature of the cytotoxicity of the D503 cells, in contrast to cytotoxic cells from the cattle immunized with buffalo-derived parasites, was the very high level of killing of the cell line infected with *T. parva parva* (Muguga) (85% cytotoxicity). Killing by cytotoxic cells from D487 [immunized with *T. parva bovis* (Boleni)] was overall lower than that by cytotoxic cells from the other cattle, being between 40 and 60% for all the infected target cell lines, except for those infected with *T. parva lawrencei* (7065), *T. parva lawrencei* (7014) and *T. parva lawrencei* (6999), for which killing was between 20 and 40%.

MHC restriction of bulk cultures of cytotoxic cells. The *Theileria*-specific bulk-cultured cytotoxic lymphocytes from animals D505 and D482 (MHC phenotype w6/KN8) and from animals D487 and D504 (MHC phenotype w6/KN12) were tested for MHC restriction by using targets cells which were infected with *T. parva parva* (Marikebuni) and which were autologous, matched for both MHC BoLA *A* locus haplotypes, matched for one haplotype, or MHC mismatched. The highest level of killing with PBM from D505 and D482 at an effector/target ratio of 40:1 was for the autologous or fully MHC-matched target cell line (98 and 65%, respectively), and the next highest level of killing was for the half-matched target cell line with the phenotype w10/KN8 (75 and 35%, respectively). After the second stimulation, there was little or no killing of the half-matched target cell line with the phenotype w6/w7 (less than 15%), and there was no killing of the mismatched target cell line with the phenotype w10, KN104/W10. The results with *Theileria*-specific cytotoxic cells from animals D487 and D504 indicated that the cytotoxic cells were restricted by KN12: the levels of killing with D487 and D505 were 65 and 43%, respectively, for the fully matched or autologous cell line and 63 and 25%, respectively, for the target cell line matched for KN12 only. There was little or no killing of target cells with the phenotype w6/w7 (18 and 10%, respectively), and there was no killing of the mismatched cell line.

Analysis of parasite strain specificities after stimulation of

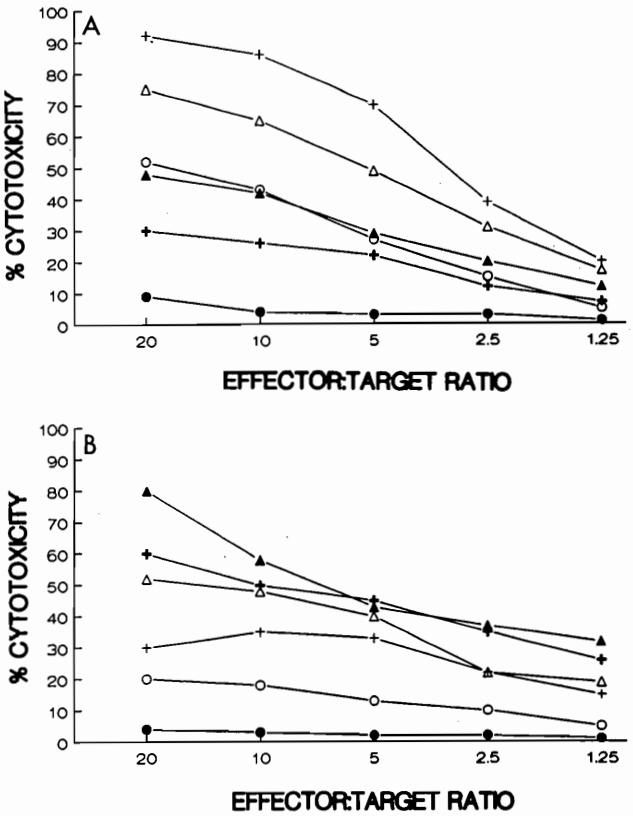

FIG. 4. Analysis of the parasite strain specificities of PBM from animal D482 after three stimulations in vitro with cells infected with *T. parva lawrencei* (7014) (A) or *T. parva parva* (Muguga) (B) and tested on autologous target cells infected with the following buffalo- or cattle-derived *T. parva* parasites: +, *T. parva lawrencei* (7014); △, *T. parva parva* (Marikebuni); ○, *T. parva lawrencei* (7013); +, *T. parva parva* (Muguga); ▲, *T. parva parva* (Uganda); and ●, none (uninfected blast cells).

cultures with cells infected with parasites other than the immunizing strain. Killing with bulk-cultured PBM from five of the immunized cattle was lowest for target cells infected with *T. parva parva* (Muguga). Further experiments were therefore conducted to determine whether common antigens existed between *T. parva parva* (Muguga)-infected cells and cells infected with buffalo-derived parasites. To do this, we stimulated PBM from animal D482 with cells infected with *T. parva parva* (Muguga) to preferentially expand any effector cells which recognized antigens shared between cells infected with *T. parva parva* (Muguga) and *T. parva lawrencei* (7014). The level of proliferation achieved was comparable to that obtained when cells infected with *T. parva lawrencei* (7014) were used to stimulate PBM from animal D482 (results not shown).

Strain specificities of cytotoxic cells generated in cultures of D482 PBM stimulated with cells infected with *T. parva parva* (Muguga) were then compared with those of cytotoxic cells generated in cultures of D482 PBM stimulated with *T. parva lawrencei* (7014). Stimulation of PBM with *T. parva parva* (Muguga)-infected cells resulted in more than 50% killing of *T. parva parva* (Muguga), *T. parva parva* (Uganda), and *T. parva parva* (Marikebuni)-infected cells but much less killing of cells infected with buffalo-derived parasites such as *T. parva lawrencei* (7014) and *T. parva lawrencei* (7013) (Fig. 4). In contrast, the effectors generated by stimulation of PBM with cells infected with *T. parva lawrencei* (7014) yielded high levels of killing of target cells infected with *T. parva lawrencei* (7014) (the immunizing as well as the stimulating strain), *T. parva lawrencei* (7013), *T. parva parva* (Marikebuni), and *T. parva parva* (Uganda) but low levels of killing of *T. parva parva* (Muguga)-infected target cells (Fig. 4). These results indicated that the antigen(s) predominantly recognized by cytotoxic cells generated by stimulation of D482 PBM with *T. parva parva* (Muguga)-infected cells was expressed at high levels on cells infected with cattle-derived parasites but at low levels on cells infected with the buffalo-derived parasites evaluated here. Furthermore, the different pattern of killing observed when D482 PBM were stimulated with the *T. parva parva* (Muguga)-infected cell line indicated that the common or cross-reactive antigens predominantly recognized by this bulk effector population were distinct from those generated when PBM were stimulated with the *T. parva lawrencei* (7014)-infected cell line.

Analysis of parasite strain specificities after stimulation of cultures with a cloned *T. parva*-infected cell line. The ability of in vitro-stimulated D482 PBM to kill cells infected with a variety of different parasite stocks may have been partially attributable to the fact that the uncloned cytotoxic cells were produced by stimulation of the PBM culture with an infected cell line which was composed of cells infected with different parasites. To test this hypothesis, we stimulated PBM from animal D482 [immunized with *T. parva lawrencei* (7014)] with a clone of D482 *T. parva lawrencei* (7014)-infected lymphocytes and compared their cytolytic activity with that of PBM stimulated with the uncloned parent *T. parva lawrencei* (7014)-infected cell line. In both cases, there was a high level of killing of the cloned *T. parva lawrencei* (7014)-infected cells when they were used as targets, and the levels of killing of target cell lines *T. parva lawrencei* (7013), *T. parva parva* (Muguga) and *T. parva parva* (Marikebuni) were the same (Fig. 5). However, the levels of killing of the uncloned *T. parva lawrencei* (7014)- and *T. parva parva* (Uganda)-infected cells were lower when cytotoxic effectors were generated by stimulation of PBM with the cloned *T. parva lawrencei* (7014)-infected cell line. This result indicated that the effector population generated by stimulation of the PBM with the cloned infected cell line was more restricted in its antigen recognition, probably because of a lack of generation of effector cells which reacted with the predominant antigen(s) expressed on a proportion of the cells within the heterogeneous *T. parva parva* (Uganda)- and *T. parva lawrencei* (7014)-infected target cell lines.

Analysis of parasite specificities of cloned effector populations. The preceding experiments suggested that more than one cytotoxic effector population reactive with cells infected with *T. parva* was found in the PBM of D482 and may have been responsible for the broad specificity of cytotoxicity that we observed with the panel of target cells. To determine if there was any single effector population which recognized an antigen common to all the target cell lines tested and which was expressed at high enough levels to result in significant cytotoxicity of the infected cells, we established clones of cytotoxic cells from the PBM of D482. Clones D482.32 and D482.38 killed all 10 infected target cell lines tested but not autologous uninfected blast cells (Fig. 6). The lowest level of killing was for *T. parva parva* (Muguga)-, *T. parva parva* (Mariakani)-, and *T. parva bovis* (Boleni)-infected cells. The patterns of cytotoxicity of two other clones, D482.35 and D483.39, tested on four target cell lines [*T. parva lawrencei* (7013 and 7014) and *T. parva parva* (Muguga and Marikebuni)] were identical to that obtained with clone D482.38.

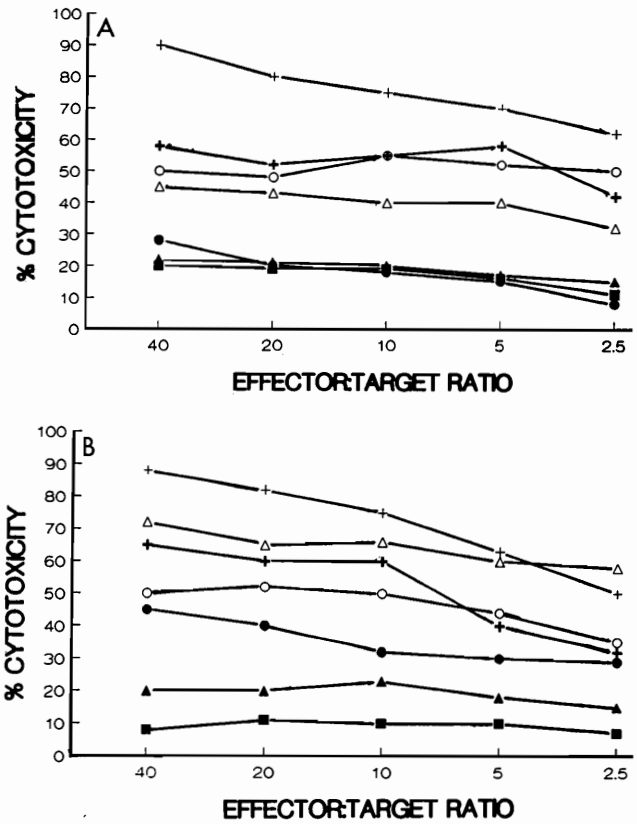

FIG. 5. Analysis of the parasite strain specificities of PBM from animal D482 after three stimulations in vitro with a clone of the *T. parva lawrencei* (7014)-infected cell line (A) or the uncloned parent *T. parva lawrencei* (7014)-infected cell line (B) and tested on autologous target cells infected with the following buffalo- or cattle-derived *T. parva* parasites: +, a clone of the *T. parva lawrencei* (7014)-infected cell line; △, *T. parva lawrencei* (7014)-infected bulk cell line; ○, *T. parva lawrencei* (7013); ▲, *T. parva parva* (Muguga); +, *T. parva parva* (Marikebuni); ●, *T. parva parva* (Uganda); and ■, none (uninfected blast cells).

The patterns of cytotoxicity obtained with these three clones were distinguished from that obtained with clone D482.32 in that the level of killing by clone D482.32 was higher for *T. parva parva* (Marikebuni)-infected target cells and lower for *T. parva lawrencei* (7013)-infected target cells.

DISCUSSION

The number of *T. parva* parasite strains is unknown, but the evidence indicates that it is potentially vast (6, 7, 17, 22), making the prospects for a subunit vaccine based on strain-specific antigens very poor. This study investigated the potential expression of common antigens which could be recognized by parasite-specific cytotoxic effector cells. To do this, we immunized cattle with one of seven parasite stocks. The stocks were selected on the basis of results from cross-immunity studies which indicated their potential for inducing immunity to infection with a number of heterologous parasite stocks (29, 31; Irvin et al., in press; S. Morzaria, personal communication). Since there is no single stock representative of *T. parva lawrencei* (7), we evaluated five different *T. parva lawrencei* stocks isolated from naturally infected buffaloes obtained from two different localities in Kenya. Characterization with antischizont MAb and repetitive-sequence DNA probes indicated differences among these five parasite stocks and heterogeneity within stocks.

To compare the specificities of immune responses within the group of animals immunized with the different parasite stocks, we minimized genetic differences among the experimental animals and phenotypic differences among the infected target cell lines as follows. (i) PBM from only two of the cattle (one each of MHC class I phenotypes w6/KN8 and w6/KN12) were used for in vitro infection with sporozoites to establish the target and stimulator cell lines. (ii) The experimental animals used for immunization were the progeny of a single bull and cow, produced by embryo transfer. These cattle therefore had a high degree of genetic relatedness and were matched for one or both alleles of the MHC class I *A* locus antigens. Differences in immune responses to *T. parva* parasites among the siblings can probably be largely attributed to the antigenicity of the parasite stock rather than to the influence of genetic differences, although the w6/KN8 cytotoxic cells were restricted largely by the KN8 determinant and the w6/KN12 cytotoxic cells were restricted by the KN12 determinant rather than by the w6 common haplotype, as hoped. Since it has been shown that the MHC phenotype of an animal influences the parasite strain specificities of its cytotoxic cells (16a), some constraints must be applied to generalizing the results.

The cytotoxic cells generated in bulk cultures of PBM from the cattle immunized with the five buffalo-derived parasite stocks were similar in their parasite strain specificities. In most instances, there was a high level of killing of target cells infected with buffalo-derived stocks and lower levels of killing of target cells infected with cattle-derived stocks, except for those infected with *T. parva parva* (Marikebuni), indicating an antigenic similarity or relatedness between these cells and those infected with the different buffalo-derived *T. parva lawrencei* parasites. The patterns of cytotoxicity generated with these uncloned populations of effector cells and the panel of heterologous target cells may have resulted from the combined effect of several effector populations, each with a distinct antigen specificity. This was demonstrated to be the case for bulk cultures of cytotoxic cells from animal D482 in two experiments performed here. In one experiment, when D482 PBM stimulated by cells infected with *T. parva parva* (Muguga)- or with *T. parva lawrencei* (7014)-infected stimulators were compared, the pattern of cytotoxicity for the target cell panel was altered, suggesting the recognition of different antigens and therefore the generation of different effector populations, depending on the stimulator cells used. In another experiment, there was a lower level of killing of the uncloned parent infected cell line when a clone of infected cells was used as the stimulator than when an uncloned, probably heterogeneous, cell line was used as the stimulator. This population of effectors either did not react with as many cells within the bulk (uncloned) infected cell line or recognized an antigen(s) which was expressed at low levels on the cell surfaces of a proportion of the cells within the bulk cell line. We concluded that the ability of the bulk-cultured PBM to kill target cells infected with a variety of different *T. parva* parasites was dependent on either stimulation with a mixed population of infected cells or stimulation with a population of infected cells not represented by the cloned infected cell line used here.

We were, however, able to demonstrate that dominant common or cross-reactive antigens were expressed on cell lines infected with various parasite stocks by generating

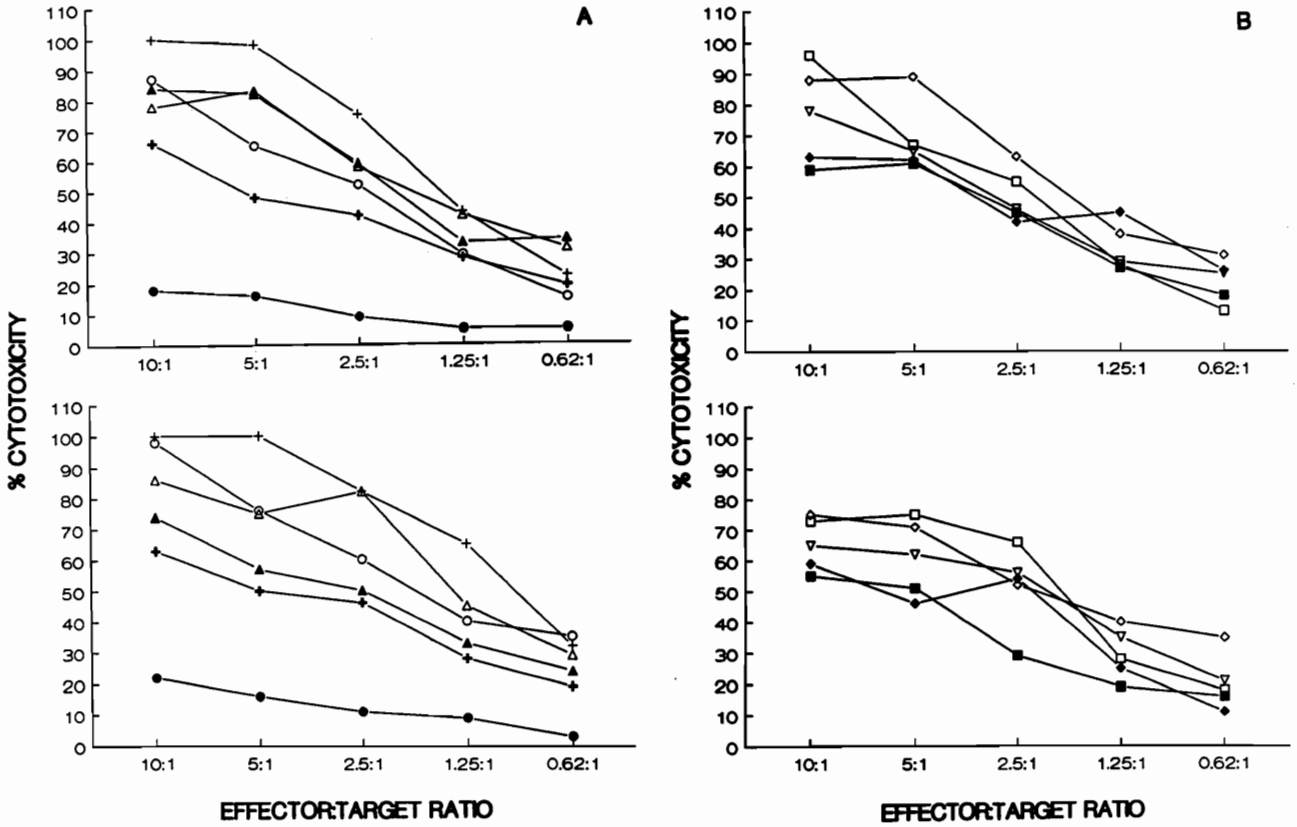

FIG. 6. Analysis of the parasite strain specificities of *Theileria*-specific cytotoxic clones from immune steer D482 stimulated with *T. parva lawrencei* (7014) and tested on autologous target cells infected with the following buffalo- or cattle-derived *T. parva* parasites: (A) +, *T. parva lawrencei* (7014); ○, *T. parva parva* (Uganda); ▲, *T. parva parva* (Marikebuni); △, *T. parva lawrencei* (7013); +, *T. parva parva* (Muguga); and ●, uninfected blasts; (B) □, *T. parva lawrencei* (7065); ▽, *T. parva lawrencei* (6999); ◇, *T. parva lawrencei* (6998); ◆, *T. parva parva* (Mariakani); and ■, *T. parva bovis* (Boleni). (Top) Clone D482.32. (Bottom) Clone D482.38.

clones of cytotoxic effector cells which had cytotoxic activity against the entire panel of infected target cell lines. The different degrees of killing of the different target cell lines by the cloned cytotoxic cells indicated either that the level of expression of the common antigen that they recognized varied among the cell lines infected with the different parasite stocks or that some of the cell lines expressed a cross-reacting rather than an identical determinant. Levels of expression of the target antigens could either vary with the cell cycle or be constitutively low on some cells. Nevertheless, partial killing of some of the infected cell lines probably was not indicative of an inability of such cytotoxic cells to inhibit the growth of these cell lines, since Morrison et al. (23) showed that cloned effectors which yielded partial killing of *T. parva parva* (Marikebuni)-infected cells were able to markedly inhibit the growth of these same cells in vitro.

The buffalo-derived *T. parva lawrencei* stocks seemed overall very effective in inducing cytotoxic cells which reacted with common antigens on the surfaces of cells infected with different parasite stocks, since all five randomly chosen stocks representing parasites endemic in at least two different geographical locations of Kenya were very similar in this respect. An effective vaccine against East Coast fever will probably have to combine more than one of the major parasite-derived antigens which are expressed on the surfaces of schizont-infected cells, since there are MHC constraints in the effectiveness of some antigen epitopes in stimulating cytotoxic T-cell responses (16a). It would obviously be advantageous to use parasite antigen epitopes which are common among cells infected with different parasite strains to reduce the total number required to yield protection against a range of parasite stocks. The clones generated during the course of this study will provide tools for identifying such antigen epitopes and the parasite genes encoding them.

ACKNOWLEDGMENTS

T.M.K. was supported by the German Academic Exchange Agency through the University of Nairobi.

We are grateful to B. A. Allsopp of the University of Cambridge, Cambridge, United Kingdom, for the use of the 623-bp *T. parva* repetitive DNA fragment.

LITERATURE CITED

1. **Allsopp, B. A., and M. T. E. T. Allsopp.** 1988. *Theileria parva*: genomic DNA studies reveal intra-specific sequence diversity. Mol. Biochem. Parasitol. **28:**77–84.
2. **Baldwin, C. L., B. M. Goddeeris, and W. I. Morrison.** 1987. Bovine T-helper clones specific for lymphocytes infected with *Theileria parva*: parasite strain specificity and class II MHC restriction. Parasite Immunol. **9:**499–513.
3. **Baldwin, C. L., M. N. Malu, and J. G. Grootenhuis.** 1988. Evaluation of cytotoxic lymphocytes and their parasite strain specificity from African buffalo infected with *Theileria parva*. Parasite Immunol. **10:**393–403.
4. **Brocklesby, D. W., S. F. Barnett, and G. R. Scott.** 1961.

Morbidity and mortality rates in East Coast fever (*Theileria parva* infection) and their application to drug screening procedures. Br. Vet. J. **117**:529–531.

5. **Brown, C. G. D.** 1979. Propagation of *Theileria*, p. 223–254. *In* K. Maramorosch and H. Hirumi (ed.), Practical tissue culture applications. Academic Press, Inc., New York.
6. **Conrad, P. A., K. Iams, W. C. Brown, B. Sohanpal, and O. Ole-MoiYoi.** 1987. DNA probes detect genomic diversity in *Theileria parva* stocks. Mol. Biochem. Parasitol. **25**:213–216.
7. **Conrad, P. A., O. K. Ole-MoiYoi, C. L. Baldwin, T. T. Dolan, C. J. O'Callaghan, R. E. G. Njamunggeh, J. G. Grootenhuis, D. A. Stagg, B. L. Leitch, and A. S. Young.** 1979. Characterization of buffalo-derived theilerial parasites with monoclonal antibodies and DNA probes. Parasitology **98**:179–188.
8. **Cunningham, M. P., C. G. D. Brown, M. J. Burridge, A. D. Irvin, I. M. Kirimi, R. E. Purnell, D. E. Radley, and G. G. Wagner.** 1974. Theileriosis: the exposure of immunised cattle in a *Theileria lawrencei* enzootic area. Trop. Anim. Health Prod. **6**:39–43.
9. **Cunningham, M. P., C. G. D. Brown, M. J. Burridge, and R. E. Purnell.** 1973. Cryopreservation of infective particles of *Theileria parva*. Int. J. Parasitol. **3**:583–587.
10. **Dolan, T. T., A. Linyonyi, S. K. Mbogo, and A. S. Young.** 1984. Comparison of long acting oxytetracycline and parvaquone in immunisation against East Coast fever by infection and treatment. Res. Vet. Sci. **37**:175–178.
11. **Dolan, T. T., D. E. Radley, C. G. D. Brown, M. P. Cunningham, S. P. Morzaria, and A. S. Young.** 1980. East Coast fever. 4. Further studies on the protection of cattle immunized with a combination of theilerial strains. Vet. Parasitol. **6**:325–332.
12. **Eugui, E. M., and D. L. Emery.** 1981. Genetically restricted cell mediated cytotoxicity in cattle immune to *Theileria parva*. Nature (London) **290**:251–254.
13. **Goddeeris, B. M., J. M. Katende, A. D. Irvin, and R. S. C. Chumo.** 1982. Indirect fluorescent antibody test for experimental and epizootiological studies on East Coast fever (*Theileria parva* infection in cattle). Evaluation of a cell culture antigen fixed and stored in suspension. Res. Vet. Sci. **33**:360–365.
14. **Goddeeris, B. M., and W. I. Morrison.** 1987. The bovine autologous *Theileria* mixed leucocyte reaction: influence of monocytes and phenotypes of the parasitized stimulator cell on proliferation and parasite specificity. Immunology **60**:63–69.
15. **Goddeeris, B. M., W. I. Morrison, and A. J. Teale.** 1986. Generation of bovine cytotoxic cell lines specific for cells infected with the protozoan parasite *Theileria parva* and restricted by products of the major histocompatibility complex. Eur. J. Immunol. **16**:1243–1249.
16. **Goddeeris, B. M., W. I. Morrison, A. J. Teale, A. Bensaid, and C. L. Baldwin.** 1986. Bovine cytotoxic T-cells specific for cells infected with the protozoan parasite *Theileria parva*: parasite strain specificity and class I major histocompatibility complex restriction. Proc. Natl. Acad. Sci. USA **83**:5238–5242.

16a. **Goddeeris, B. M., W. I. Morrison, D. G. Toye, and R. Bishop.** 1990. Strain specificity of bovine *Theileria parva*-specific cytotoxic T cells is determined by the phenotype of the restricting class I MHC. Immunology **69**:39–44.

17. **Irvin, A. D., D. A. E. Dobbelaere, D. M. Mwamachi, T. Minami, P. R. Spooner, and J. G. R. Ocama.** 1983. Immunization against East Coast fever: correlation between monoclonal antibody profiles of *Theileria parva* stocks and cross immunity *in vivo*. Res. Vet. Sci. **35**:341–346.

17a. **Irvin, A. D., S. P. Morzaria, F. C. Munatswa, and R. A. I. Norval.** 1989. Immunization of cattle with a *Theileria parva bovis* stock from Zimbabwe protects against challenge with virulent *T. p. parva* and *T. p. lawrencei* stocks from Kenya. Vet. Parasitol. **32**:271–278.

18. **Jordt, T., G. D. Mahan, B. N. Toyray, W. K. Ngulo, W. I. Morrison, J. Rawle, and M. Murray.** 1986. Successful transfer of N'Dama embryoes into Boran recipients. Vet. Rec. **119**:246–247.
19. **Kurtti, J. F., U. O. Munderloh, A. D. Irvin, and G. Buscher.** 1980. *Theileria parva*: early events in the development of bovine lymphoblastoid cell lines persistently infected with macroschizonts. Exp. Parasitol. **52**:280–290.
20. **Lalor, P. A., W. I. Morrison, B. M. Goddeeris, R. M. Jack, and S. J. Black.** 1986. Monoclonal antibodies identify phenotypically and functionally distinct cell types in the bovine lymphoid system. Vet. Immunol. Immunopathol. **13**:121–140.
21. **Lawrence, J. A., and P. K. I. MacKenzie.** 1980. Isolation of a non-pathogenic *Theileria* of cattle transmitted by *Rhipicephalus appendiculatus*. Zimb. Vet. J. **11**:27–35.
22. **Minami, T., P. R. Spooner, A. D. Irvin, J. G. R. Ocama, D. A. E. Dobbelaere, and T. Fujinaga.** 1983. Characterisation of stocks of *Theileria parva* by monoclonal antibody profiles. Res. Vet. Sci. **35**:334–340.
23. **Morrison, W. I., B. M. Goddeeris, and A. J. Teale.** 1987. Bovine cytotoxic T cell clones which recognize lymphoblasts infected with two antigenically different stocks of the protozoan parasite *Theileria parva*. Eur. J. Immunol. **17**:1703–1709.
24. **Morrison, W. I., B. M. Goddeeris, A. J. Teale, C. M. Croocock, S. J. Kemp, and D. A. Stagg.** 1987. Cytotoxic T cells elicited in cattle challenged with *Theileria parva* (Muguga): evidence for restriction by class I MHC determinants and parasite strain specificity. Parasite Immunol. **9**:563–578.
25. **Newson, J., J. Naessens, D. A. Stagg, and S. J. Black.** 1986. A cell surface antigen associated with *Theileria parva lawrencei*-infected bovine lymphoid cells. Parasite Immunol. **8**:149–158.
26. **Pearson, R. W., R. S. Hewett, G. E. Roelants, D. A. Stagg, and T. T. Dolan.** 1982. Studies on the induction and specificity of cytotoxicity to *Theileria*-transformed cell lines. J. Immunol. **128**:2509–2513.
27. **Radley, D. E., C. G. D. Brown, M. J. Burridge, M. P. Cunningham, I. M. Kirimi, R. E. Purnell, and A. S. Young.** 1975. East Coast fever. 1. Chemoprophylactic immunization of cattle against *Theileria parva* (Muguga) and five theilerial strains. Vet. Parasitol. **1**:35–41.
28. **Radley, D. E., C. G. D. Brown, M. P. Cunningham, C. D. Kimber, F. L. Musisi, R. C. Payne, R. E. Purnell, D. A. Stagg, and A. S. Young.** 1975. East Coast fever. 3. Chemoprophylactic immunization of cattle using oxytetracycline and a combination of theilerial strains. Vet. Parasitol. **1**:51–60.
29. **Radley, D. E., A. S. Young, C. G. D. Brown, M. J. Burridge, M. P. Cunningham, F. L. Musisi, and R. E. Purnell.** 1975. East Coast fever. 2. Cross immunity trials with a Kenya strain of *Theileria lawrencei*. Vet. Parsitol. **1**:43–50.
30. **Teale, A. J., S. J. Kemp, A. S. Young, and R. L. Spooner.** 1983. Selection of major histocompatibility type (BoLA) of lymphoid cells derived from a bovine chimaera and transformed by *Theileria* parasites. Parasite Immunol. **5**:329–333.
31. **Young, A. S., C. G. D. Brown, M. J. Burridge, M. P. Cunningham, I. M. Kirimi, and A. D. Irvin.** 1973. Observations on the cross-immunity between *Theileria lawrencei* (Serengeti) and *Theileria parva* (Muguga) in cattle. Int. J. Parasitol. **3**:723–728.