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Phenotypic characterization of *Theileria parva* schizonts by two-dimensional gel electrophoresis*

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Abstract. Biosynthetically radiolabelled *Theileria parva* schizonts were purified from bovine lymphoblastoid cells and their proteins were analyzed by two-dimensional gel electrophoresis and autoradiography. The protein spot patterns of schizont proteins from three stocks of *T. parva parva* indicated that the phenotypic diversity among the stocks was minimal, with the Mariakani and Uganda stocks being identical and the Muguga stock showing only a few differences in minor spots. Comparison of the spot patterns of schizonts of three *T. parva* subspecies showed that *T. p. parva* and *T. p. bovis* differed in only one protein and thus could not be reliably distinguished on the basis of their protein differences. However, *T. p. lawrencei* showed several protein differences and could be distinguished easily from the other subspecies. Differences in schizont-protein spot patterns were also seen when two different cell lines were infected with the same *Theileria* stabilate, when one cell line was infected with two different stabilates of the same stock and when uncloned and cloned infected cell lines were used. These results suggest the possibility that selection of phenotypically different parasites could occur in vivo or in vitro.

Theileria parva is a tick-borne protozoan parasite of domestic cattle and African buffalo (*Syncerus caffer*) in East and Central Africa. Three subspecies of *T. parva* are distingushed by clinical, behavioral and epidemiological features of the parasites (Uilenberg 1981), although this distinction is more for convenience than for scientific accuracy. *T. p. parva* causes an acute disease in cattle known as East Coast fever (ECF), whereas *T. p. bovis* produces a generally mild infection in cattle (Rhodesian fever). The third subspecies, *T. p. lawrencei*, is a buffalo-derived parasite that causes an acute and fatal disease in cattle (Corridor disease), in which there are fewer detectable parasites than are seen in ECF. These three subspecies are indistinguishable by morphological or serological criteria.

Considerable effort has been devoted to developing methods of characterizing *T. parva* parasites, including the use of anti-schizont monoclonal antibodies in indirect immunofluorescence (Minami et al. 1983) and immunoblotting procedures (Shapiro et al. 1987), isoenzyme analysis (Melrose and Brown 1979; Melrose et al. 1980, 1984; Musisi et al. 1981; Allsopp et al. 1985) and hybridization with *T. parva*-specific DNA probes (Conrad et al. 1987a; Allsopp and Allsopp 1988).

Studies in which cattle were immunized by infection and treatment (Radley 1978; Irvin et al. 1983) have shown that there are several antigenically different stocks of *T. parva* that do not induce cross-protective immune responses in cattle. Cell-mediated immune responses, in particular *Theileria*-specific cytotoxic T cells, are operative during the immunization and challenge of immune animals, and their specificity correlates with in vivo cross-protection results obtained with some parasite stocks (Morrison et al. 1986, 1987).

Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) has been shown to be a powerful method for resolving and characterizing

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proteins in complex biological materials (Anderson and Anderson 1978a, b) and has been used for analysis of the proteins of trypanosomes (Anderson et al. 1985; Pearson and Anderson 1983) and malarial parasites (Tait 1981; Howard et al. 1983). In the present study, purified schizonts of different stocks and subspecies of *T. parva* were analysed by 2D-PAGE and autoradiography to determine whether 2D-PAGE could be used to characterize *T. parva* and to identify proteins that differ between stocks and might be possible target molecules for cell-mediated immune responses.

Materials and methods

Theileria culture. The *Theileria*-infected bovine lymphoblastoid cell lines used in this study are listed in Table 1. Cloned T lymphoblastoid cells 657.G6 and T19.4 were established and maintained in vitro as previously described by Brown and Grab (1985) and Morrison et al. (1987), respectively. The cell lines were infected in vitro as previously described by Dobbelaere et al. (1984), with theilerial sporozoites obtained either from ticks in the same batch, such as those used to prepare the bulk stabilates 3087, 3014, 3029, 3066, 3039 and 3084, or from adult ticks that fed as nymphs on cattle infected with stabilates 836 or 2245, as indicated in Table 1. Infected cell lines were cultured in RPMI 1640 medium (Gibco Ltd.; Paisley, UK) with 25 m*M* *N*-2-hydroxyethyl piperazine-*N*-2-ethanesulfonic acid (HEPES) containing 10%–20% (v/v) heat-inactivated foetal bovine serum (Hyclone; Logan, Utah, USA) supplemented with 2 m*M* L-glutamine and 50 μg/ml gentamycin.

The *Theileria*-infected cells were cloned by limiting dilution into microtiter wells containing confluent bovine foetal thymic fibroblasts (Kurtti et al. 1981) at concentrations calculated to give 10, 3, 1 or 0.3 *Theileria*-infected cells/well. Clones were selected for propagation and testing from dilutions for which the number of wells showing growth was <15%.

Biosynthetic labelling of cells with [^{35}S]-methionine. After centrifugation of cultivated *Theileria*-infected lymphoblastoid cells at 200 *g* for 10 min, the cells were resuspended to a concentration of 10^6/ml in 10 ml RPMI 1640 medium containing 0.75 μg/ml methionine, dialysed heat-inactivated foetal bovine serum, 2 m*M* L-glutamine, 50 μg/ml gentamycin, 10 m*M* HEPES and 200 μCi [^{35}S]-methionine (>1,000 Ci/mmol, SJ204, Amersham International; Buckinghamshire, UK). The mixture was dispensed into a 25-cm^2 tissue-culture falsk, gassed with 5% (v/v) CO_2 in air and incubated at 37° C for 18 h in a horizontal position.

Purification of schizonts. Schizonts were purified according to Sugimoto et al. (1988). The radiolabelled cells were treated with Ah-1 hemolysin (Asao et al. 1984) at room temperature for 25 min in the presence of Ficoll 400 (Pharmacia Fine Chemicals; Uppsala, Sweden). The concentrations of the hemolysin and Ficoll 400 used for schizont purification from each cell line are given in Table 1. The cell lysate was layered onto 45% and 65% (v/v) Percoll (Pharmacia) solutions in a tube, and the sample was centrifuged in an ultracentrifuge with an SW-41 rotor (Beckman Instruments Inc.; Calif, USA) for 30 min at 85,000 *g*. Schizonts were collected from a band at the interface between the 45% and 65% Percoll solutions.

Sample preparation for electrophoresis. Purified schizonts were treated with pH 9.5 solubilization buffer, 9 *M* urea, 4% (v/v) NP40, 2% (v/v) 2-mercaptoethanol and 2% (v/v) ampholine (pH 9–11, LKB; Bromma, Sweden) at room temperature for 15 min as previously described by Anderson et al. (1985). Samples were centrifuged at 13000 *g* for 30 s in a microcentrifuge (Eppendorf Gerätebau; Hamburg, FRG) and the supernatant was transferred to a fresh microcentrifuge tube and stored at −70° C until use.

To determine the amount of radioactivity precipitable with trichloroacetic acid, a 2-μl sample was placed on a filter paper strip, air-dried and placed in boiling 10% (w/v) trichloroacetic acid for 10 min. The filter paper was washed with distilled water, methanol and acetone and dried under a heat lamp. The radioactivity was determined in a liquid scintillation counter (LS6800, Beckman Instruments Inc.; Calif, USA).

2D-PAGE. Solubilized schizont supernatants were thawed and centrifuged at 13000 *g* for 30 s immediately prior to 2D-PAGE.

Table 1. *Theileria*-infected bovine lymphoblastoid cell lines and the concentrations of Ficoll 400 and Ah-l hemolysin used for schizont purification

Bovine host cell	Parasite stock	Sporozoite stabilate number	Clone	Concentration of hemolysin and Ficoll used for schizont purification	
				Ficoll 400 (%[w/v])	Ah-l hemolysin (μg/ml)
657.G6	*T. p. parva* (Muguga)	836	uncloned	2	23
657.G6	*T. p. parva* (Muguga)	836	2C2.2C11	2	23
657.G6	*T. p. parva* (Muguga)	3087[a]	uncloned	2	11.5
657.G6	*T. p. parva* (Marikebuni)	2245	2C3.C12.C12	2	23
T19.4	*T. p. parva* (Marikebuni)	3014[b]	uncloned	2	23
T19.4	*T. p. parva* (Marikebuni)	2245	clone 1	2	23
657.G6	*T. p. parva* (Mariakani)	3029	uncloned	2	23
657.G6	*T. p. parva* (Uganda)	3066	uncloned	2	23
657.G6	*T. p. bovis* (Boleni)	3039	uncloned	2	11.5
657.G6	*T. p. lawrenceia* (Buffalo 7014)	3084	uncloned	5	69

[a] Stabilate 3087 was prepared from ticks fed on a bovid inoculated with stabilate 836
[b] Stabilate 3014 was prepared from ticks fed on a bovid inoculated with stabilate 2245

For each sample, 5×10^5 counts/min radioactivity were applied to an isoelectric focusing gel in the ISO-DALT system (Anderson and Anderson 1978a, b). Ampholines used in the first-dimension tube gels were a 1:2 mixture of pH 4–6 and pH 3.5–10 Ampholines (LKB; Bromma, Sweden). Gels with a 7.5%–16.5% (w/v) acrylamide gradient were used for the second dimension. The Ampholine mixture and second-dimension gradients were empirically optimized to give a good dispersion and resolution of protein spots. Gels were fixed, treated with 3% glycerol, dried under vacuum at 80° C and autoradiographed for 2–4 weeks using X-ray film (RX-100, Fuji Film Co.; Tokyo, Japan). Prior to photography, film backgrounds were partially removed by clearing of the non-exposed surface with household bleach.

Comparison of 2D-PAGE gel patterns. Autoradiograms of two-dimensional (2D) gel patterns were compared by placing one autoradiograph over another on an X-ray light box. Superimposition of gel spot constellations enabled the detection of qualitative differences in gel spots and, in some cases, the detection of quantitative differences. This simple procedure was accurate and reliable because the ISO-DALT multiple 2D gel system allowed us to run ten gels (poured from the same batch of reagents) simultaneously; thus, all samples to be compared could be electrophoresed in one run. Interpretations of gel spot patterns were done using previously published criteria (Anderson and Anderson 1979; Pearson and Anderson 1983).

For each schizont preparation, radiolabelling of *Theileria*-infected cells and subsequent schizont purification were repeated twice, and for each radiolabelled sample 2D-PAGE was carried out at least twice. Additionally, a sample of [^{35}S]-methionine-labelled proteins of schizonts purified from 657.G6 lymphoblastoid cells infected with *T. p. parva* (Muguga, clone 2C2.2C11) was run in each batch as a control.

Results

Comparison of T. parva stocks

2D gel autoradiograph patterns of schizont proteins from the 657.G6 cell line infected with the Muguga, Mariakani or Uganda stocks of *T. p. parva* were compared (Fig. 1 A–C). All major spots and most of the minor spots were found to be conserved among the parasites in these stocks. Four minor protein spots were found in schizonts from the Muguga stock (Fig. 1 A, two spots each in boxes 1 and 3) that were not observed in the parasites from the Mariakani and Uganda stocks. Three protein spots were seen in schizonts from the Mariakani and Uganda stocks (Fig. 1 B, C; one spot each in boxes 2, 11 and 12) that were not found in schizonts from the Muguga-infected cell

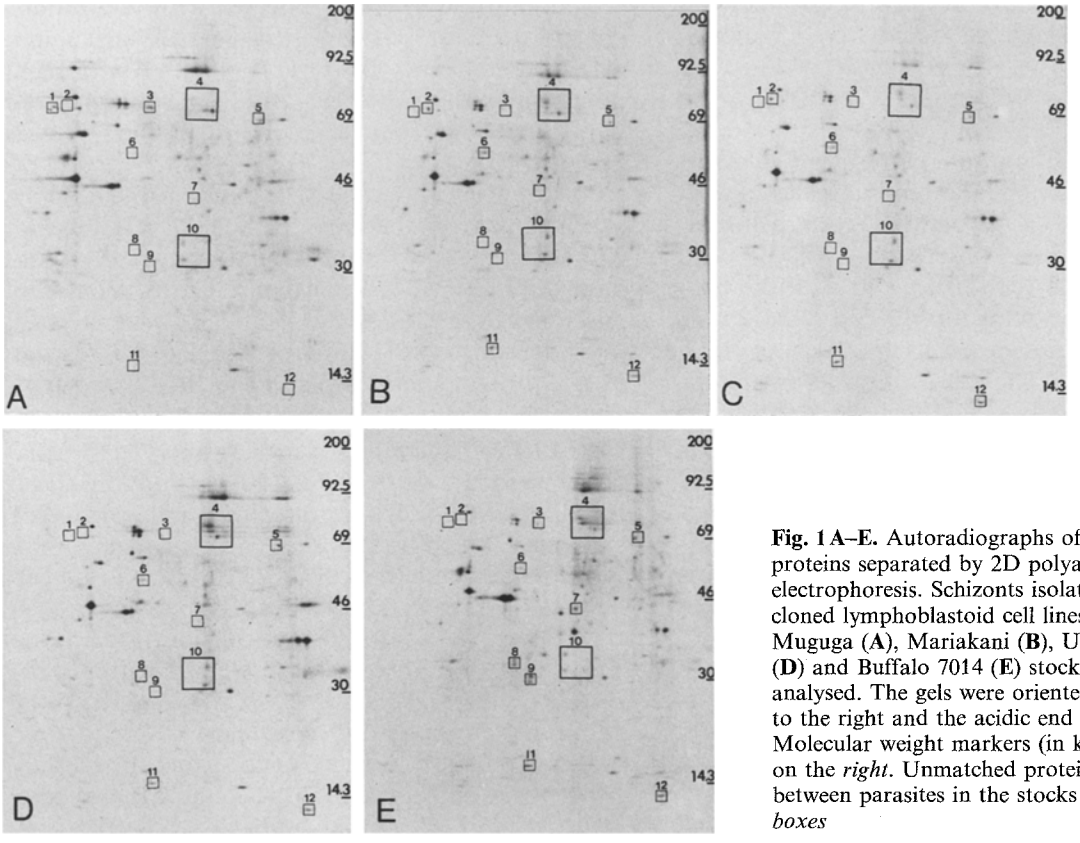

Fig. 1 A–E. Autoradiographs of *T. parva* schizont proteins separated by 2D polyacrylamide gel electrophoresis. Schizonts isolated from the 657.G6 cloned lymphoblastoid cell lines infected with Muguga (**A**), Mariakani (**B**), Uganda (**C**), Boleni (**D**) and Buffalo 7014 (**E**) stocks of *T. parva* were analysed. The gels were oriented with the basic end to the right and the acidic end to the left. Molecular weight markers (in kDa) are indicated on the *right*. Unmatched protein spots that differ between parasites in the stocks are indicated by *boxes*

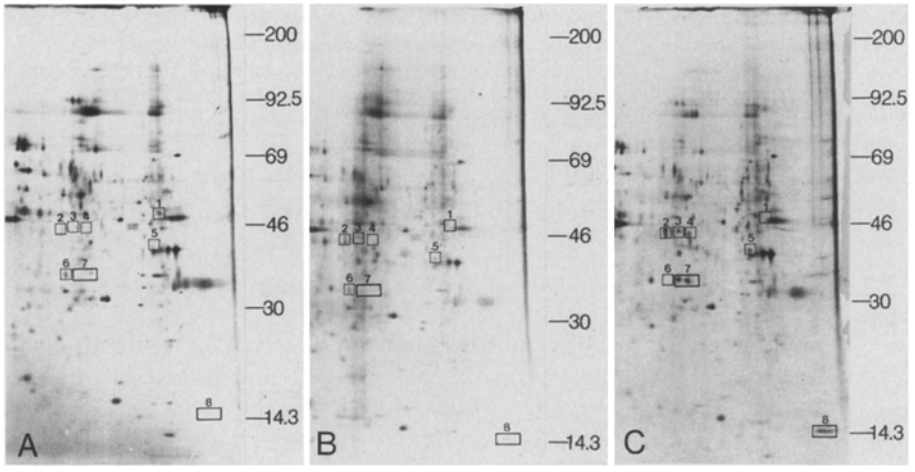

Fig. 2A–C. Autoradiographs of *T. p. parva* (Marikebuni) schizont proteins separated by 2D polyacrylamide gel electrophoresis. [^{35}S]-methionine-labelled proteins of schizonts isolated from the T19.4 cloned lymphoblastoid cell lines infected with sporozoite stabilate 2245 (**A**) and 3014 (**B**) and the 657.G6 cloned lymphoblastoid cell lines infected with sporozoite stabilate 2245 (**C**) were analysed. The gels were oriented with the basic end to the right and the acidic end to the left. Only the basic half of the gels is shown because no differences were found in the acidic half. Molecular weights (in kDa) are indicated on the *right*. Unmatched protein spots are indicated by *boxes*

line. Differences were also observed between schizonts from the Muguga-infected cell line and those from the other cell lines in one protein spot in box 4 and two protein spots in box 10. The proteins in box 10 may be charge-shifted forms of the same proteins (more acidic in Muguga schizonts). The 2D gel spot patterns of schizonts from the Mariakani and Uganda stocks were identical.

Comparison of T. parva subspecies

The 2D gel autoradiograph patterns of proteins in schizonts from the 657.G6 cell line infected with the Boleni stock (a *T. p. bovis* stock) and Buffalo 7014 (a *T. p. lawrencei*-type stock) (Fig. 1D, E) were compared with each other and with the protein patterns of the schizonts from the Muguga, Mariakani and Uganda stocks (*T. p. parva*-type stocks; Fig. 1A–C). The 2D spot patterns of proteins from schizonts in the Boleni stock (Fig. 1D) were identical to those of proteins in schizonts from the Mariakani and Uganda stocks (Fig. 1B, C), except for one minor spot (box 2), which was absent in the Boleni stock. Parasites from the buffalo 7014 stock expressed five schizont proteins that were not seen in 2D gels of schizonts from any of the other stocks (one spot in box 7 and two spots each in boxes 8 and 9). In addition, there was a charge shift in one protein spot in the buffalo-derived schizonts, compared with the other schizonts (box 5), and two spots in box 10 were superimposed on spots in schizonts from the Muguga stock (Fig. 1A, E; box 10). The last three spot differences (boxes 5 and 10) could be charge-shifted forms of the same proteins.

Comparison of Marikebuni stocks

The 2D protein spot pattern of *T. p. parva* (Marikebuni) schizonts derived from the T19.4 cloned lymphoblastoid cell lines infected with two different sporozoite stabilates of *T. p. parva* (Marikebuni stabilates 2245 and 3014) were compared with each other and with the pattern of *T. p. parva* (Marikebuni) schizonts from the 657.G6 cell line infected with stabilate 2245.

There were differences in minor protein spots in schizonts from the same cell line (T19.4) infected with different sporozoite stabilates of the Marikebuni stock (Fig. 2A, B) and in schizonts from two different cell lines (T19.4 and 657.G6) infected with the same sporozoite stabilates (Fig. 2A, C). A total of five protein spots (two spots in box 2, one spot each in boxes 3, 5, 8) were found in schizonts from the T19.4 cell line infected with *T. p. parva* (Marikebuni stabilate 3014; Fig. 2B) but not in those from the same cell line infected with a different stabilate (2245, Fig. 2A). Two protein spots in box 1 were found only in schizonts from the T19.4 cell line infected with stabilate 2245 (Fig. 2A).

Protein spot patterns of schizonts from the T19.4 and 657.G6 cell lines infected with sporozoite stabilate 2245 (Fig. 2A, C) differed in four additional protein spots (one spot in box 4, one in box 6 and two in box 7). The same spot differences observed between Figs. 2A and 2B were also seen.

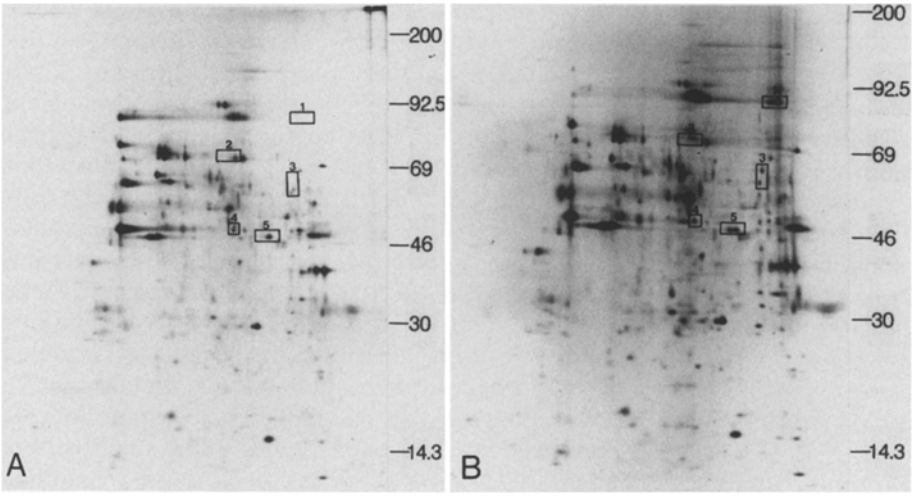

Fig. 3A, B. Autoradiographs of 2D-PAGE profiles of *T. p. parva* (Muguga) schizont proteins from cloned and uncloned cell lines. [^{35}S]-methionine-labelled proteins of schizonts isolated from the 657.G6 lymphoblastoid cell line infected with sporozoite stabilate 836 of the *T. p. parva* (Muguga) stock and cloned twice by limiting dilution (**A**) were compared with proteins of schizonts from the uncloned infected cells (**B**). The gel was oriented with the basic end to the right and the acidic end to the left. Molecular weights (in kDa) are sown on the *right*. Unmatched protein spots are indicated by *boxes*

Comparison of the schizonts from uncloned and cloned cells

The 2D protein spot pattern of schizonts purified from the *T. p. parva* (Muguga)-infected 657.G6 cell line, which was cloned twice after sporozoite infection (Fig. 3A), was compared with that of schizonts purified from the uncloned cell line (Fig. 3B). Four protein spots (two in box 1 and one each in boxes 2 and 5) were observed in the pattern from schizonts of the uncloned cell line, which were absent in the schizonts from the cloned cell line. Two protein spots (one in box 2 and a doublet in box 4) that did not occur in the parasites from the uncloned cells were observed in the schizonts from the cloned cell line. A possible molecular weight shift was observed in one protein spot in box 3.

Discussion

Over 200 *T. parva* schizont proteins could be seen by 2D-PAGE and autoradiography of schizont proteins that were biosynthetically labelled with ^{35}S-methionine. The reproducibility of the 2D gel patterns of schizont proteins was good, with almost identical results obtained in separate experiments. As a separate control, the [^{35}S]-methionine-labelled proteins of schizonts from a twice-cloned cell line *T. p. parva* (Muguga)-infected 657.G6 cells were used in each ISO-DALT run to monitor the resolution and reproducibility of the 2D patterns. The run-to-run reproducibility was excellent as judged from the protein autoradiograph patterns.

Two cloned lymphoblastoid cell lines, 657.G6 and T19.4, which were infected with different parasite stocks, were also analysed to investigate the effect of host cells on schizont protein expression as well as the possibility of host-cell protein contamination in the schizont preparations.

Comparisons of the 2D gel patterns of schizont proteins from three stocks of *T. p. parva* indicated that phenotypic diversity among theilerial stocks was restricted to minor proteins. The 2D gel patterns of the Mariakani and Uganda stocks were identical, whereas their protein patterns differed in several minor spots from the Muguga stock.

Comparison of the 2D gel protein patterns of schizonts from three stocks of *T. p. parva* with those of schizonts from a *T. p. bovis* (Boleni) stock showed that the Boleni, Mariakani and Uganda stocks were almost identical, with only a single spot difference between the three patterns. Thus, with these three stocks it appeared that *T. p. parva* and *T. p. bovis* cannot be distinguished easily on the basis of their protein patterns. On the other hand, the 2D gel patterns of the buffalo-derived *T. p. lawrencei* stock showed several protein differences from all three *T. p. parva* stocks and from the *T. p. bovis* stock. Further stocks of *T. p. lawrencei* need to be analysed to determine whether the protein patterns can be used to discriminate this subspecies from the others.

Differences in the 2D gel protein patterns were observed between schizonts from two different cell lines (657.G6 and T19.4) infected with the same stabilate (2245) of *T. p. parva* (Marikebuni). Similar differences were found in the protein patterns between schizonts from the same T19.4 cells infected with two different stabilates (2245 and 3014) of *T. p. parva* (Marikebuni). Differences in 2D gel patterns between schizonts from different stabi-

lates were also observed using *T. p. parva* (Muguga)-infected cells (data not shown). One possible explanation for these differences is that phenotypically different parasites existing in a theilerial stock might be selected during in vitro infection, culturing and cloning of host cells. Alternatively, the selection may occur in vivo, as the stabilate 3014 of *T. p. parva* (Marikebuni) had been prepared from an animal infected with stabilate 2245. The possibility that in vitro selection of phenotypically different parasites within a stabilate could occur is supported by the fact that there were spot differences in the 2D patterns between schizonts from uncloned infected cells and cells cloned after infection with *T. p. parva*.

The heterogeneity detected within the same stabilate (2245) of the Marikebuni stock may have major implications for the interpretation of cross-immunization trials using the Marikebuni stock and analysis of cytotoxic T-cell responses. The Marikebuni stock provides protection against challenge with the Muguga and many other stocks (Irvin et al. 1983; Mutugi et al. 1989) as well as challenge in the field (Morzaria et al. 1987). Recent data with cytotoxic T-cell clones on cloned target cells infected with the same isolate have shown that some cloned Marikebuni targets are killed and others are not (Goddeeris and Morrison, in preparation).

Antigenically heterogeneous populations within theilerial stocks have been demonstrated by indirect immunofluorescence using anti-schizont monoclonal antibodies (Shiels et al. 1986; Conrad et al. 1987b). In addition, genetic heterogeneity of *T. p. parva* stocks was suggested after analysis using DNA probes (Conrad et al. 1987a). Our results support these findings. The occurrence of mixed parasite populations is an inherent problem in the characterization of *Theileria* species. Although cloned *T. parva*-infected cell lines are available, cloning of *Theileria*-infected cells does not mean that the parasite itself is cloned, since it is theoretically possible that more than one sporozoite may invade a single host cell during in vivo and in vitro infection (Irvin 1987).

Our results suggest that it may be possible to distinguish buffalo-derived *T. p. lawrencei* parasites from the other *T. parva* subspecies; this needs to be investigated further with larger numbers of isolates. Furthermore, a technique for producing monospecific antibodies against minor proteins separated by 2D-PAGE, as previously reported by Chiles et al. (1987), might be useful in raising antibody reagents that potentially could distinguish *T. parva* subspecies. Differences in 2D gel protein patterns between *T. p. parva* and *T. p. bovis* subspecies are too minor and too variable to distinguish accurately between these subspecies, which are classified on the basis of clinical and epidemiological features. This implies that clinical, epidemiological and immunological differences seen with different parasite stocks reflect subtle differences in the protein composition of the intracellular schizonts. Only minor differences in protein profiles were detected between different stocks of *T. p. parva*. Since the important effector mechanism influencing immunity to East Coast fever is thought to be major histocompatibility complex-restricted cytotoxic T-cell killing of the schizont-infected lymphocytes (reviewed by Morrison et al. 1986), small differences in *Theileria* peptides can easily be accommodated into current models of T-cell recognition and used to explain differences in the induction and maintenance of immunity.

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