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Expression of Tac Antigen Component of Bovine Interleukin-2 Receptor in Different Leukocyte Populations Infected with *Theileria parva* or *Theileria annulata*

D. A. E. DOBBELAERE,[1]†* T. D. PROSPERO,[2] I. J. RODITI,[1]‡ C. KELKE,[1] I. BAUMANN,[1] M. EICHHORN,[1] R. O. WILLIAMS,[1] J. S. AHMED,[3] C. L. BALDWIN,[4]§ H. CLEVERS,[5] AND W. I. MORRISON[4]||

Kernforschungszentrum Karlsruhe, Institut für Genetik und Toxikologie, Postfach 3640, D-7500 Karlsruhe,[1] Zentrum für Molekulare Biologie, Heidelberg,[2] and Institut für Parasitologie und Tropenveterinärmedizin der Freien Universität Berlin, Berlin,[3] Federal Republic of Germany; International Laboratory for Research on Animal Diseases, Nairobi, Kenya[4]; and Klinische Immunologie, Akademisch Ziekenhuis Utrecht, Utrecht, The Netherlands[5]

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The Tac antigen component of the bovine interleukin-2 receptor was expressed as a Cro–β-galactosidase fusion protein in *Escherichia coli* and used to raise antibodies in rabbits. These antibodies were used for flow cytofluorimetric analysis to investigate the expression of Tac antigen in a variety of *Theileria parva*-infected cell lines and also in three *Theileria annulata*-infected cell lines. Cells expressing Tac antigen on their surface were found in all *T. parva*-infected cell lines tested whether these were of T- or B-cell origin. T cells expressing Tac antigen could be $CD4^-$ $CD8^-$, $CD4^+$ $CD8^-$, $CD4^-$ $CD8^+$, or $CD4^+$ $CD8^+$. Tac antigen expression was observed both in cultures which had been maintained in the laboratory for several years and in transformed cell lines which had recently been established by infection of lymphocytes in vitro with *T. parva*. Northern (RNA) blot analysis demonstrated Tac antigen transcripts in RNA isolated from all *T. parva*-infected cell lines. Three *T. annulata*-infected cell lines which were not of T-cell origin were also tested. Two of them expressed Tac antigen on their surface. Abundant Tac antigen mRNA was detected in these *T. annulata*-infected cell lines, but only trace amounts were demonstrated in the third cell line, which contained very few Tac antigen-expressing cells. In all cell lines tested, whether cloned or uncloned, a proportion of the cells did not express detectable levels of Tac antigen on their surface. This was also the case for a number of other leukocyte surface markers. In addition, we showed that the interleukin-2 receptors were biologically functional, because addition of recombinant interleukin-2 to cultures stimulated cell proliferation. Recombinant interleukin-2 treatment also resulted in increased amounts of steady-state Tac antigen mRNA. The relevance of interleukin-2 receptor expression on *Theileria*-infected cells is discussed.

East Coast fever is an acute and fatal disease of cattle which places major constraints on the development and expansion of the livestock industry in large parts of east and central Africa (32, 43). The causative agent, *Theileria parva*, is a sporozoan parasite which is transmitted by ticks. When infected ticks feed on susceptible cattle, mature *Theileria* sporozoites are inoculated with the tick saliva and enter leukocytes by a process known as passive endocytosis (23). Within the host cell, sporozoites differentiate into schizonts. At the same time, infected cells undergo lymphoblastoid transformation and multiplication, resulting in rapid clonal expansion and high levels of parasitosis in the lymphoid tissues of the infected animal (21, 42). Cell lines can be established from *Theileria*-infected cells isolated by biopsy from infected lymphoid organs (38). Once a cell line is established, cells divide every 16 to 28 h. Because host cell and parasite replicate synchronously, approximately 95% of the cells in a culture harbor the parasite. Infected cell lines can also be generated in vitro by infecting bovine lymphocytes with mature sporozoites harvested from *T. parva*-infected ticks (9). With this technique, it has been shown that *T. parva* sporozoites have the ability to invade a variety of cell types, such as granulocytes, monocytes, and lymphocytes. Neither monocytes nor granulocytes give rise to infected cell lines, however, and only lymphoid cells will establish continuously growing *T. parva*-infected cell lines (43). Although *T. parva* was originally thought to transform only T lymphocytes (47), it has recently been shown that bovine $CD4^+$ and $CD8^+$ T cells and B cells and $CD4^-$ $CD8^-$ null cells can all become transformed by infection with *T. parva* (4, 34–35; P. A. Lalor, Ph.D. thesis, Brunel University, Uxbridge, United Kingdom, 1983). The cells described as null cells have recently been shown to be γδ T-cell receptor-expressing T lymphocytes (15).

It has been shown previously that growth of *T. parva*-infected cells occurs via an autocrine mechanism (10, 17) involving the secretion of a growth factor with interleukin-2 (IL-2)-like activity. In addition, it was shown that cells from a cloned *T. parva*-infected T-cell line, TpM(803), constitutively express functional IL-2 receptors (IL-2R) on their surface (16, 17). IL-2R expression was shown to be strictly dependent on the continuous presence of the parasite in the

* Corresponding author.
† Present address: Institute for Animal Pathology, University of Berne, Länggass-Strasse 122, CH-3012 Berne, Switzerland.
‡ Present address: Institute for General Microbiology, University of Berne, Baltzerstrasse 4, CH-3012 Berne, Switzerland.
§ Present address: Department of Microbiology, The Ohio State University, Columbus, OH 43210-1292.
|| Present address: AFRC Institute for Animal Health, Compton, Newbury, Berkshire RG16 0NN, United Kingdom.

TABLE 1. FCF analysis of antigen expression by *Theileria*-infected cell lines

Cell line (age of culture)	Differentiation antigen expression[a]								
	CD2	CD4	CD8	CD5	CD6	sIg[b]	WC1	MHC class II	IL-2R/Tac
Tp (Muguga)	+	+/−	−	+/−	+/−	−	+	+	+/−
Tp (Kilifi)	ND[c]	+/−	+/−	−	+/−	−	+/−	+	+/−
T1	+	+	+/−	−	+	−	+/−	+	+/−
T4	+	−	+	−	+	−	+	+	+/−
N1	+	−	+/−	−	−	−	+	+	+/−
N2	+	−	−	−	−	−	+	+	+/−
B1	−	−	−	−	−	+/−	−	+	+/−
B2	−	−	−	−	−	+/−	−	+	+/−
IN3 (10 wk)	+	+	+/−	+	+	−	−	+/−	+/−
IN7 (10 wk)	+	−	+/−	+/−	+/−	−	+/−	+/−	+/−
IN9 (10 wk)	+	+	−	+	+	−	+/−	+	+/−
IN10 (10 wk)	+	+/−	−	+/−	+/−	−	+/−	+/−	+/−
Ta288	+/−	+/−	−	−	−	−	+/−	+	+/−
Ta363	+/−	−	−	−	−	−	−	+	+/−
Ta(Ä)	+/−	−	−	−	−	+	+/−	+	+/−
ConA-treated LNC	+	+/−	+/−	−	+/−	+/−	+/−	+/−	+/−

[a] Symbols: +, >99% of cells were positive; −, >99% of cells were negative; +/−, both positive and negative cells present.
[b] sIg, Surface immunoglobulin.
[c] ND, Not determined.

host cell cytoplasm, since elimination of the parasite by the theilericidal drug BW720c (31) led to the rapid disappearance of IL-2R expression (16, 17).

The constitutive expression of functional IL-2R on the surface of *T. parva*-infected cells probably confers a selective advantage to the parasite-infected cells. It was therefore important to examine whether constitutive IL-2R expression was a general characteristic of all *T. parva*-infected cell lines. We analyzed a range of cloned and uncloned *T. parva*-infected cell lines of different origins and phenotypes. We also tested cell lines infected with the closely related parasite *T. annulata*, the causative agent of tropical theileriosis, which has been reported to transform a different subset of mononuclear cells (53, 54). The continued growth of these cells also depends on the continuous presence of the parasite in their cytoplasm (49).

MATERIALS AND METHODS

***T. parva*- and *T. annulata*-infected cell lines.** All cell lines were cultured at 37°C in either Dulbecco L15 or RPMI 1640 medium (both from GIBCO, Uxbridge, United Kingdom) supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum (GIBCO), 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer (pH 7.1), L-glutamine (20 μg/ml), benzylpenicillin (100 U/ml), streptomycin sulfate (100 μg/ml), and 5×10^{-5} M 2-mercaptoethanol. These media are referred to hereafter as cL15 and cRPMI, respectively.

The characteristics and maintenance of the cloned *T. parva*-infected T-cell line TpM(803) have been described elsewhere (17). The cloned T-cell line Tp(Kilifi) was established from cells isolated by lymph node biopsy from a cow infected with *T. parva* (geographical stock; Kilifi).

The infected cell lines T1, T4, B1, B2, N1, and N2 were established at the International Laboratory for Research on Animal Diseases, Nairobi, as described previously (15). In brief, bovine peripheral blood mononuclear cells from animal D(409) were separated by flow sorting (FACStar-plus; Becton Dickinson Co., Sunnyvale, Calif.) into three populations on the basis of expression of surface CD2, immunoglobulin M (IgM), and the determinant for monoclonal antibody (MAb) IL-A29. The latter antibody reacts specifically with a population of lymphocytes which do not express surface immunoglobulin and are negative for the bovine lymphocyte differentiation markers CD2, CD4, and CD8 (15). The sorted cells were then infected by incubation with *T. parva* (Muguga) sporozoites (12, 18) isolated from infected ticks. Cells were then dispensed at limiting dilution into microtiter plates, and cell cultures growing at the clonal level were expanded. A summary of the phenotypes of these cell lines is presented in Table 1.

The cell lines IN3, IN7, IN9, and IN10 were generated by infecting bulk cultures of concanavalin A (ConA)-stimulated bovine lymph node cells (LNC), prepared as described by Mastro and Pepin (39), with *T. parva* (Muguga) sporozoites (obtained from the Institut für Parasitologie und Tropenveterinärmedizin der Freien Universität Berlin). Cells (5×10^7) were incubated for 30 min with salivary gland homogenates prepared from 30 *T. parva*-infected ticks that had fed on rabbits for 4 days (18). Cells were then cultured in 25-cm^2 Falkon flasks at 5×10^5 cells per ml in 10 ml of cRPMI and monitored for signs of transformation, characterized by IL-2-independent growth and the appearance of parasites in the cell cytoplasm (18). Cultures in which parasitized cells were detected were propagated by passage every 3 to 4 days. Approximately 10 weeks after infection, cultures had attained a growth rate comparable to that of other *T. parva*-infected cell lines, and ≥95% of the cells contained the parasite in their cytoplasm.

T. annulata-infected cell lines were generated as described previously (28) and cultured in cRPMI. The cell lines Ta288 and Ta363 originated from bovine peripheral blood leukocytes, isolated from animals 288 and 363 (Institut für Parasitologie und Tropenveterinärmedizin der Freien Universität Berlin) which were infected with *T. annulata* (Ankara). The *T. annulata*-infected cell line Ta(Ä) was obtained from G.

Büscher (Tierärztliche Hochschule, Hannover, Federal Republic of Germany [FRG]) and is infected with the parasite *T. annulata* (Ägypten).

Northern (RNA) blot analysis. Northern blot analysis of polyadenylated RNA isolated from the various cell lines was performed as described before (19). The following ^{32}P-labeled probes were used, as indicated: a bovine IL-2R/Tac antigen cDNA probe (57); a bovine CD3γ probe (15; C. L Baldwin, H. Clevers, and K. P. Iams, Abstr. 2nd Int. Vet. Immunol. Symp. 1989, Immunobiol. Suppl. 4, p. 229), and a bovine actin cDNA probe kindly provided by A. Ehrfeld (Kernforschungszentrum Karlsruhe, Institut für Genetik und Toxikologie, Karlsruhe, FRG).

To investigate the induction of Tac antigen mRNA expression by recombinant IL-2 (rIL-2), the cell lines IN9 and IN10 were cultured in the presence or absence of 10 U of human rIL-2 ([Ala^{125}]interleukin 2; Amersham; code ARN.7010) per ml for 20 h, and polyadenylated RNA was isolated.

Expression of bovine IL-2R/Tac as a fusion protein in *E. coli* and production of polyclonal antibodies. A 2.3-kb *Hin*dIII-*Eco*RI fragment of the bovine IL-2R/Tac cDNA clone (from base 206 to the end of the cDNA clone) (57) was repaired by filling in and subcloned in the *Sma*I site of the bacterial expression vector pEX3 (55). Plasmids containing the construct in the correct orientation were identified by digestion with *Pst*I and *Kpn*I. *E. coli* POP2136 (a gift from O. Raibaud, Institut Pasteur, Paris, France) were transformed with the construct. Expression in *E. coli*, isolation of the fusion protein, and immunization of rabbits were carried out as described previously (51).

Immunoprecipitation of bovine IL-2R/Tac antigen. TpM (803) cells or ConA-stimulated bovine LNC (10^7 per sample) were metabolically labeled by incubating them for 3 h in 4 ml of methionine-free RPMI containing 600 μCi of ^{35}S-labeled methionine (L-[^{35}S]methionine; Amersham code SJ1015; specific activity, 1,000 Ci/mmol; 1 mCi = 37 MBq). Cells were washed once in phosphate-buffered saline, and cytoplasmic protein extracts were prepared as follows. Cells were lysed for 5 min in 100 μl of ice-cold CE buffer (10 mM HEPES [pH 7.9], 60 mM KCl, 1 mM EDTA, 0.5% Nonidet P-40, 1 mM dithiothreitol [DTT], 1 mM phenylmethylsulfonyl fluoride [PMSF]). Cell debris and nuclei were removed by centrifugation at 4°C for 5 min at 1,200 × *g*; 50 μl of NE buffer (250 mM Tris [pH 7.8], 60 mM KCl, 1 mM EDTA, 1 mM DTT, 1 mM PMSF) was added to the supernatant, which was then further cleared by centrifugation at 100,000 × *g* for 30 min at 4°C. Preimmune serum (5 μl) or anti-Tac antigen immune serum (5 μl) was incubated at 4°C for 1 h with 75 μl of a 50% (vol/vol) protein A-Sepharose suspension in TSA buffer (10 mM Tris [pH 8.0], 140 mM NaCl, 0.025% sodium azide) and for a further 2 h in the presence of 25 μg of bovine serum albumin. The antibody-protein A-Sepharose slurry was then washed three times in 300 μl of a 2:1 mixture of CE and NE buffers. After the last wash, 80 μl of CE-NE (2:1) buffer and 150 μl of cytoplasmic cell extract were added to the antibody-protein A slurry, which was left to incubate for 3 h at 4°C. The slurry was washed twice with 500 μl of TSA buffer containing 0.1% Triton X-100, once with 500 μl of TSA buffer, and twice with 500 μl of 50 mM Tris (pH 6.8). After the last wash, 12 μl of sample buffer (2% sodium dodecyl sulfate, 10% glycerol, 80 mM Tris [pH 6.8], 2% mercaptoethanol) was added, and the samples were boiled for 3 min and separated on a 10% sodium dodecyl sulfate–polyacrylamide electrophoresis gel. The gel was then dried and exposed for autoradiography for 2 days.

Analysis of the surface of *Theileria*-infected cell lines. Bovine cell lines infected with *T. parva* or *T. annulata* were examined for the expression of a panel of lymphocyte surface markers by indirect immunofluorescence staining and flow cytofluorimetry (FCF) analysis on a FACStar-Plus. All incubations and washes were done at 0 to 4°C. Mouse MAbs specific for the following markers were used: IL-A12 (specific for bovine CD4 [7]); IL-A17 (specific for bovine CD8 [20]); B5/4 (specific for bovine surface IgM [46]); IL-A29 (Workshop Nomenclature: Workship Cluster 1, WC1; a marker specific for γδ T cells [15, 41]); DAKO-HLA-DR (reacts with a monomorphic determinant on major histocompatibility complex [MHC] class II molecules; Dakopatts, Hamburg, FRG); CC17 (specific for bovine CD5 [30]); IL-A26 (specific for bovine CD2 [5]); and IL-A27 (specific for bovine CD6 [6]). For these antibodies, the second reagent was affinity-purified goat anti-mouse immunoglobulin fluorescein conjugate (Becton Dickinson, Heidelberg, FRG).

To monitor Tac antigen expression, different *Theileria*-infected cell lines were analyzed by FCF with immune serum (diluted 1:200) from a rabbit immunized with the Cro–β-galactosidase–IL-2R/Tac fusion protein. In this case the second reagent was affinity-purified goat anti-rabbit immunoglobulin fluorescein conjugate (Dianova, Hamburg, FRG). FCF analysis was done on a FACStar-Plus/CONSORT 30 cytometer system; a Lexel 90-4 argon laser provided excitation light (200 mW, 488 nm) in the light control mode. Fluorescence signals were measured as pulse area (integral) because of the large size of many parasitized cells. Conventional computer gating was used on red fluorescence (after counterstaining with propidium iodide [5 μg/ml]) to exclude dead cells and on forward versus right-angle light scatters to exclude debris and aggregates. On control samples (second antibody only), a gate was set on a histogram of green fluorescence to give a maximum "positive" value of 0.3 to 0.5%. In test samples, all cells falling above this value were scored as positive.

Proliferation assay. *Theileria*-infected cells were seeded at various dilutions (1×10^3 to 5×10^4 cells per ml) in the absence or presence of 10 U of rIL-2 per ml. Proliferation was monitored by pulse-labeling with [^{3}H]thymidine for 2 h as described before (19).

RESULTS

Characterization of antibodies directed against bovine IL-2R/Tac antigen. Incubation at 37°C of log-phase cultures of *E. coli* containing IL-2R/Tac expression vector pIRX9 led to the production of a fusion protein (data not shown), which was isolated from preparative polyacrylamide gels and used as the immunogen for the production of antibodies against the bovine Tac antigen. Serum from a rabbit immunized with the Tac antigen fusion protein was assayed by immunoprecipitation and by FCF analysis for reactivity with the bovine Tac antigen. For this purpose, the cloned *T. parva*-infected T-cell line TpM(803), which has previously been shown to express Tac antigen mRNA and to respond to rIL-2 by enhanced proliferation, was used (16, 17). In parallel, bovine LNC stimulated with the mitogen ConA for 3 days were also analyzed. These cells express abundant Tac antigen mRNA and also respond to rIL-2 (16, 17).

Immunoprecipitation of cytoplasmic extracts of [^{35}S]methionine-labeled TpM(803) or ConA-stimulated bovine LNC was carried out with either anti-Tac antigen immune serum or preimmune serum from the same rabbit. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the

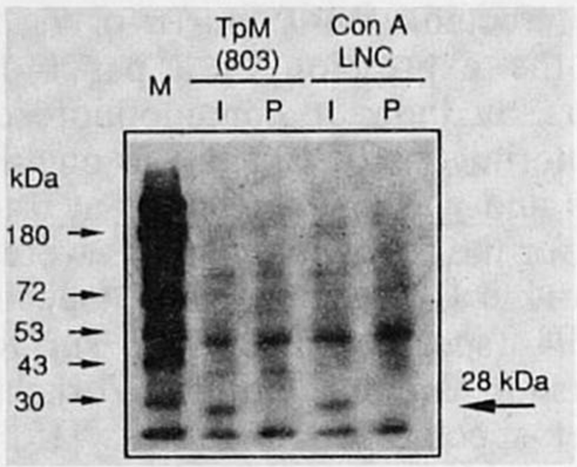

FIG. 1. Immunoprecipitation of Tac antigen with rabbit antiserum to the Tac antigen fusion protein. *T. parva*-infected TpM(803) cells and ConA-stimulated LNC were biosynthetically labeled with [^{35}S]methionine for 3 h, and immunoprecipitation was carried out on cytoplasmic extracts with either immune (I) or preimmune (P) serum. The immunoprecipitates were analyzed on a 10% sodium dodecyl sulfate–polyacrylamide gel. The arrow indicates a 28-kDa molecule which was specifically precipitated by immune serum.

immunoprecipitated products revealed a protein with an apparent molecular mass of approximately 28 kDa which was specifically precipitated by the immune serum (Fig. 1). This protein was detected in cytoplasmic extracts of TpM(803) cells as well as in ConA-stimulated LNC, and its molecular mass corresponds to that of the unmodified form of the IL-2R/Tac antigen peptide (28.7 kDa) predicted from the amino acid sequence (57). A number of other proteins were seen which were precipitated in an unspecific manner by preimmune as well as immune serum. However, these proteins did not interfere with the analysis.

Extensive posttranslational modifications of the Tac antigen involving N- and O-linked glycosylation and sulfation have been described (25). The 28-kDa unmodified peptide and not the mature 55-kDa Tac antigen was immunoprecipitated probably because the cells were processed immediately after metabolic labeling for a relatively short time compared with the total time required to process mature Tac antigen. Alternatively, it is possible that the serum-free medium in which the cells were labeled did not allow posttranslational processing of the Tac antigen to its mature form.

FCF analysis also showed that anti-Tac antibodies reacted specifically with the surface of unfixed TpM(803) cells and ConA-stimulated T cells (Fig. 2). Although TpM(803) is a cloned cell line, not all TpM(803) cells expressed detectable levels of Tac antigen on their surface. Cells negative for surface Tac antigen expression were consistently observed in all cell lines tested, irrespective of whether cells were growing logarithmically or had reached density arrest (data not shown). Immunofluorescence microscopy with anti-Tac antibodies also revealed differences in fluorescence intensity between single cells, ranging from very bright fluorescence to fluorescence which was barely stronger than or indistinguishable from background fluorescence. Large multinucleate *T. parva*-infected cells often showed bright surface fluorescence, indicating a high density of Tac antigen molecules. Culturing *Theileria*-infected cells in the presence of the naphthoquinone derivative BW720c results in the killing and elimination of the parasite from the host cell cytoplasm (31). We have previously shown that parasite elimination resulted in a reduction in the amount of steady-state Tac antigen mRNA expression within 3 to 5 days (16). FCF analysis of BW720c-treated cells showed that surface expression of Tac antigen also disappeared within 5 days of exposure of the cells to the drug.

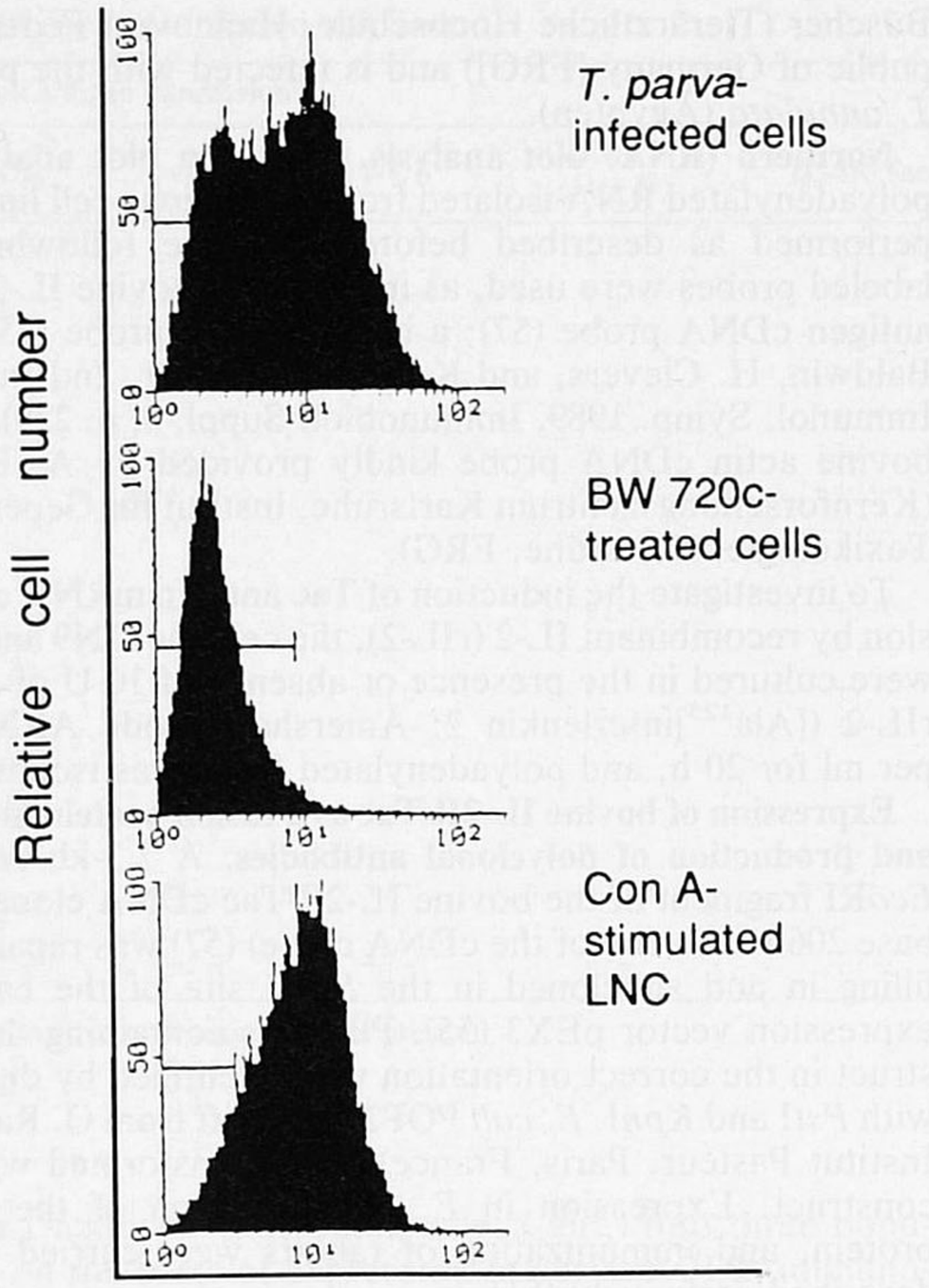

FIG. 2. FCF analysis with rabbit anti-Tac antigen antibodies. The *T. parva*-infected T-cell line TpM(803) was cultured in the absence or presence of the theilericidal drug BW720c. Uninfected ConA-stimulated bovine LNC were also analyzed. The horizontal axes show the log relative fluorescence intensity, and vertical axes indicate the relative cell number.

Expression of surface IL-2R/Tac antigen in a variety of *T. parva*-infected cell lines. Eleven different *T. parva*-infected cell lines were analyzed by FCF for the expression of bovine Tac antigen and a number of other differentiation antigens. The results are summarized in Table 1. Not only the typical T cell lines TpM(803), Tp (Kilifi), T1, and T4 but also the γδ T-cell receptor-expressing T-cell lines N1 and N2 as well as the B-cell lines B1 and B2 expressed the bovine Tac antigen. The general pattern of Tac expression for the different cell lines was the same as observed for the cell line TpM(803); only a subset of the cells were positive for Tac antigen. The percentage of positive cells varied between the different cell lines but sometimes also fluctuated between different experiments, and when tested at another time point, significant changes in the distribution of positive and negative populations were seen (data not shown). These fluctuations were observed in cloned as well as in uncloned cell lines and were also observed for other surface differentiation antigens (Table 1). Because of these variations, the data in Table 1 are presented as >99% positive cells, mixed, and >99% negative cells for the marker tested. A comparative FCF analysis of various cell lines carried out at the same time point showed that in one B-cell line, B1, 12% of the cells were negative for Tac; in the second B-cell line, B2, however, 32% of the cells were negative. In the γδ T-cell line N1, 20% of the cells were negative, whereas 44% were negative in the cell line N2. In the T-cell lines Tp (Kilifi), T1, and T4, 67, 49,

and 78% of the cells, respectively, did not express detectable levels of Tac antigen on their surface.

We also investigated whether Tac antigen expression was restricted to cell lines which had been kept in culture for several years, such as the Tp (Muguga) and Tp (Kilifi) lines, or whether cells recently transformed by infection with *T. parva* also expressed Tac antigen. All bulk cell lines established by in vitro infection of ConA-stimulated bovine LNC with *T. parva* sporozoites showed Tac antigen expression. A phenotypic analysis of four such cell lines, tested 10 weeks after infection with sporozoites, is shown in Table 1. At that time complete transformation was observed, characterized by an infection rate of ≥95%. All four cell lines showed T-cell characteristics, expressing high levels of bovine CD2; three cell lines expressed CD4; the fourth cell line was negative for CD4 but expressed CD8; one cell line expressed both CD4 and CD8.

A comparative analysis of a long-term culture of ConA-stimulated LNC from which the IN cell lines were generated is shown in Table 1. Whereas the ConA-stimulated LNC were negative for CD5, all four IN cell lines originating from this culture expressed CD5. The differentiation marker WC1 was expressed on all of the *T. parva*-infected cell lines tested except those that were of B-cell origin.

The observations described above strongly suggest that expression of the Tac antigen component of the IL-2R is a general feature of cell lines generated by infection with *T. parva* in vitro and is independent of their phenotype. The cloned cell line Tp (Kilifi), which was established from cells isolated from an infected animal, also expressed Tac antigen. Both cell lines grown as bulk cultures or as cloned cell lines expressed IL-2R/Tac.

Expression of IL-2R/Tac antigen on *T. annulata*-infected cells. *T. parva* and *T. annulata* have been shown to infect and transform different bovine peripheral blood mononuclear cells. It has been reported that T cells do not become transformed by *T. annulata*, but cell lines can be derived from infections of B cells or monocytes. Upon infection, however, B cells and monocytes appear to stop expressing surface IgM and monocyte markers, respectively (53, 54). In addition, it has been shown that Fc and C3 receptors also disappear from the surface of bovine lymphocytes upon infection with *T. annulata* (2).

Cell lines infected with *T. annulata* were analyzed for the expression of various bovine lymphocyte surface markers as well as Tac antigen. Three examples of *T. annulata*-infected cell lines which expressed Tac antigen are shown in Table 1. Once again the proportion of cells positive for Tac antigen varied between the cell lines. In this analysis, 94% of the Ta288 cells and 65% of the Ta363 cells were positive for Tac antigen. In the cell line Ta(Ä) only 6% of the cells expressed detectable levels of Tac antigen. As has been shown for all other *Theileria*-infected cell lines, the *T. annulata*-infected cell lines expressed MHC class II antigens on their surface. All three cell lines, however, also expressed CD2, and Ta288, in addition, expressed the differentiation marker CD4. The cell lines Ta(Ä) and Ta288, but not Ta363, also expressed WC1 determinants, and Ta(Ä) cells expressed surface immunoglobulin.

IL-2R/Tac antigen mRNA expression in *Theileria*-infected cell lines. To investigate IL-2R/Tac gene expression in *Theileria*-infected cells, we analyzed mRNA isolated from various *T. parva*- and *T. annulata*-infected cell lines. Tac antigen transcripts were demonstrated for all *T. parva*-infected cell lines examined. Figure 3 shows a Northern blot hybridized with a bovine IL-2R/Tac cDNA probe, using RNA isolated from TpM(803) and the six cloned *T. parva*-infected cell lines of T- and B-cell origin. Tac antigen mRNA was clearly demonstrated in all of these cell lines, including the two B-cell lines. CD3γ is a protein belonging to the T-cell receptor complex, and its expression is T-cell specific (14). When filters were rehybridized with a bovine CD3γ cDNA probe, CD3γ mRNA was only detected in cells of T-cell origin, not, as expected, in the B-cell lines.

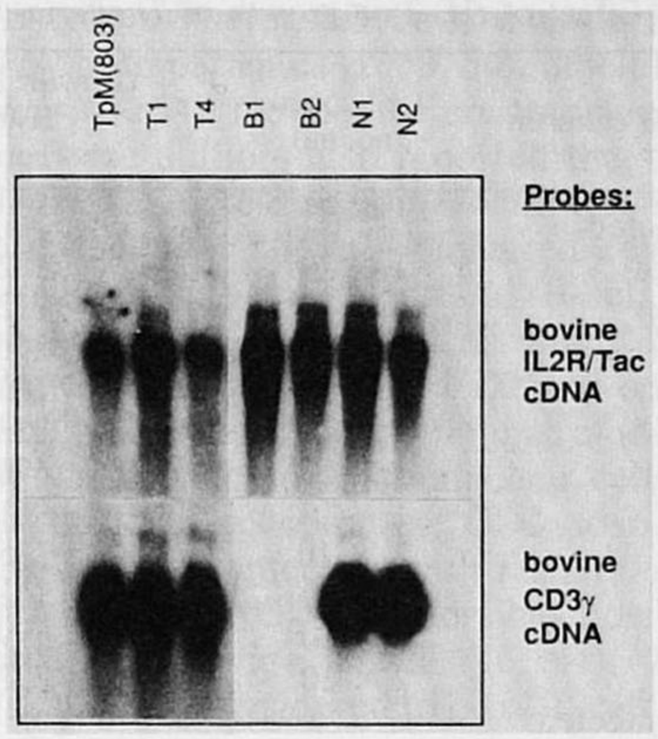

FIG. 3. Expression of Tac antigen mRNA in *T. parva*-infected cells of T- and B-cell origin. The cell lines TpM(803), T1, and T4 are of T-cell origin and express the αβ T-cell receptor; the cell lines B1 and B2 are of B-cell origin, and the lines N1 and N2 are T cells expressing the γδ T-cell receptor. Four micrograms of polyadenylated RNA was loaded per lane and analyzed by Northern blot analysis. The filter was probed sequentially with a ^{32}P-labeled bovine IL-2R/Tac cDNA probe and a bovine CD3γ cDNA probe. Films were overexposed in order to confirm the absence of CD3γ transcripts in the two B-cell lines.

Tac antigen transcripts could also be shown in the *T. annulata*-infected cell lines Ta288 and Ta363 but were barely detectable in Ta(Ä) (Fig. 4). The low levels of Tac antigen transcript observed in Ta(Ä) correlate well with the small number of Tac antigen-expressing cells detectable by FCF analysis. When filters were rehybridized with the CD3γ cDNA probe, transcripts were found in RNA isolated from the two control T-cell lines TpM(803) and T4 but not in RNA

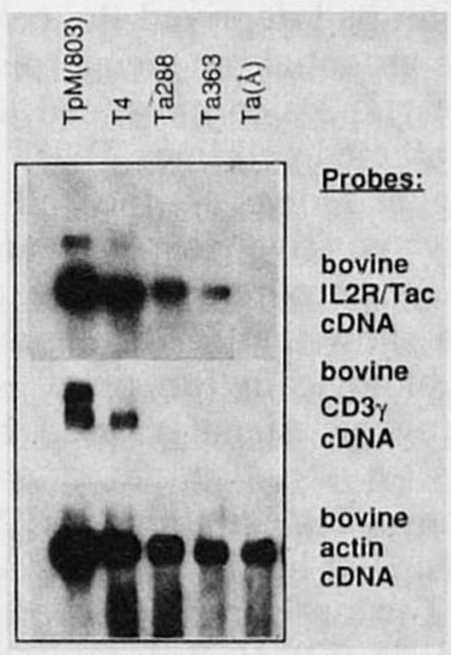

FIG. 4. Tac antigen mRNA expression in *T. annulata*-infected cell lines. Northern blot analysis of polyadenylated RNA isolated from three different *T. annulata*-infected cell lines [Ta288, Ta363, and Ta(Ä)] and two control *T. parva*-infected cell lines [TpM(803) and T4]. Filters were hybridized sequentially with cDNA probes specific for bovine IL-2R/Tac, CD3γ, and actin.

TABLE 2. Effect of rIL-2 on growth of *Theileria*-infected cells

Cell line (age of culture)	Cell concn (no./ml)	Growth[a] (% of control [^{3}H]thymidine incorporation ± SD)
T. parva infected		
Tp (Muguga)	1×10^3	152 ± 18
IN3 (10 wk)	5×10^4	212 ± 5
IN7 (10 wk)	5×10^4	256 ± 6
IN9 (10 wk)	5×10^4	163 ± 1
IN10 (10 wk)	5×10^4	223 ± 2
T1	1×10^4	490 ± 28
T4	1×10^4	961 ± 32
B1	2×10^4	135 ± 15
B2	2×10^4	153 ± 20
N1	2×10^3	199 ± 27
N2	2×10^3	227 ± 38
T. annulata infected		
Ta288	1×10^3	401 ± 78
Ta363	1×10^3	339 ± 24
Ta(Ä)	2×10^3	109 ± 15

[a] Percentage of incorporation by cells cultured without rIL-2.

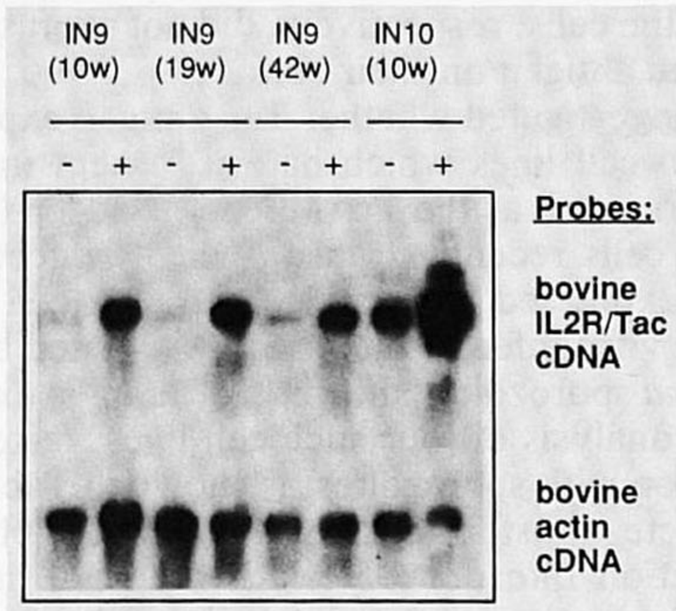

FIG. 5. Increase in Tac antigen mRNA in *T. parva*-infected cells following treatment with rIL-2. The *T. parva*-infected cell lines IN9 and IN10 were cultured in the presence (+) or absence (−) of 10 U of human rIL-2 per ml for 20 h, and polyadenylated RNA was assayed by Northern blot analysis. Filters were sequentially hybridized with cDNA probes specific for bovine IL-2R/Tac and actin. The numbers in parentheses indicate the age of the culture, expressed as weeks after transformation by infection with *T. parva*.

from the three *T. annulata*-infected cell lines tested. Filters were also rehybridized with a bovine actin cDNA probe as a control for the amount of RNA loaded for each sample (Fig. 4, lower panel). Abundant actin transcripts were demonstrated in RNA from all cell lines.

Effect of rIL-2 on growth of *Theileria*-infected cells. We have previously shown that the addition of rIL-2 to the culture medium of the *T. parva*-infected cell line TpM(803) enhanced proliferation (17). This effect was most clearly seen when cells were seeded at suboptimal density, e.g., 10^3 to 10^4 cells per ml rather than 10^5 cells per ml. We tested various *Theileria*-infected cell lines for their response to rIL-2 (Table 2). All *T. parva*-infected cell lines, including the B-cell lines, responded to the addition of 10 U of rIL-2 per ml by an increase in proliferation rate. The extent of the response varied markedly between the different cell lines but also between different experiments. Different culture conditions, in particular the cell density before passage, strongly influenced the magnitude of the IL-2 responses (data not shown). The newly established cell lines IN3, IN7, IN9, and IN10 proved very sensitive to dilution and grew very poorly unless rIL-2 was added. On some occasions, rIL-2 was not able to support the growth of very diluted cultures, indicating that the dilution had deprived the cells of factors other than IL-2 that are essential for their proliferation.

The addition of rIL-2 also enhanced the proliferation of the *T. annulata*-infected cell lines Ta288 and Ta363, which express Tac antigen. However, the cell line Ta(Ä), which was only weakly positive for Tac antigen expression, showed no significant proliferation in response to rIL-2.

The above data show that IL-2R expressed on *Theileria*-infected cells are biologically functional, since they are able to convey a mitogenic signal caused by the binding of exogenous IL-2.

Effect of rIL-2 on IL-2R/Tac antigen mRNA expression in *T. parva*-infected cells. IL-2 has been reported to upregulate the transcription of Tac antigen mRNA in both human and murine cells (8, 25, 48, 52). IL-2 also upregulates Tac antigen mRNA expression in bovine ConA-stimulated LNC and in the *T. parva*-infected cell line TpM(803), which has been grown in the laboratory for many years (19). In the newly established IN cell lines, Tac antigen mRNA was also upregulated when the cells were grown for 20 h in the presence of 10 U of rIL-2 per ml prior to RNA isolation. Figure 5 shows a Northern blot analysis of RNA isolated from the cell lines IN9 and IN10. Three different time points after infection are shown for the cell line IN9. A marked increase in the steady-state amount of Tac mRNA was observed in cells that were cultured in the presence of rIL-2. The signals are weaker than those obtained in normal blots because the filter was exposed for a shorter time in order to accentuate the difference in amounts of Tac transcript induced by treatment with rIL-2. Hybridization with a bovine actin cDNA probe showed that the difference in IL-2R/Tac-specific signals was not the result of large variations in the amount of RNA analyzed per lane.

DISCUSSION

We have produced polyclonal antibodies against the bovine Tac antigen, expressed as a fusion protein in *E. coli*, which were used to monitor Tac antigen expression on the surface of *Theileria*-infected cells. Both FCF and Northern blot analyses showed that infection with *T. parva* results in continuous Tac antigen expression in all cell lines tested regardless of their phenotype. In two of the three *T. annulata*-infected cell lines tested, Tac antigen was also expressed at levels similar to those observed in *T. parva*-infected cells. The fact that *Theileria*-infected cells can respond to rIL-2 by increased growth and by increased IL-2R/Tac gene transcription also proves that the IL-2R is biologically functional.

In general, a functional IL-2R consists of two components: (i) the low-affinity Tac antigen (α-chain, 55 kDa), which on its own is incapable of signal transduction, and (ii) an intermediate-affinity β-chain (75 kDa), which is constitutively expressed and can transduce signals. Resting T cells do not express Tac antigen, and under normal circumstances Tac antigen expression in activated lymphocytes is only a transient event (reviewed in reference 25). Upon activation, the induced Tac antigen associates with the β-chain to form a heterodimeric high-affinity IL-2R. When IL-2, secreted by activated T cells, binds to the high-affinity receptor, a signal transduction pathway is activated which usually results in cell proliferation. Binding of IL-2, however, also leads to internalization of the IL-2R–IL-2 complex and so contrib-

utes to the downregulation of IL-2R expression. Without renewed stimulation, functional IL-2R disappears, and the activated T cell returns to a resting state (13, 25). We have shown that *T. parva*-infected cells differ from normal T cells in that the IL-2R/Tac gene is constitutively transcribed and the internalized IL-2R is continuously replaced by free receptors (16). Upon infection with *Theileria* spp., the cells do not require further exogenous mitogenic stimulation (such as that provided by ConA) in order to maintain IL-2R expression. We have previously shown that the transcription factor NF-κB, which is involved in the regulation of IL-2R/Tac gene expression and is normally activated upon antigenic or mitogenic stimulation, is constitutively activated in *T. parva*-infected cells (33). The mechanism by which NF-κB activation is sustained in *Theileria*-infected cells, however, is still unknown.

Although it was originally assumed that IL-2R were expressed only on T lymphocytes, various lines of evidence have shown that activated murine and human B cells can also express IL-2R which, according to criteria such as number of receptors per cell, high- and low-affinity distribution, and mediation of growth promotion, appear to function in the same way as IL-2R on activated T cells (36, 40, 50, 56, 58). Our data show that *T. parva*-infected bovine B cells also express IL-2R and, in common with activated human or murine B cells, respond to IL-2 by enhanced proliferation.

In all cell lines, cloned or uncloned, a proportion of the cells did not express detectable levels of Tac antigen. Within one culture, the percentage of IL-2R-expressing cells was not constant. The reason for this unequal distribution of Tac antigen expression is not known, but it does not appear to be cell cycle related because it was also observed in cultures that had reached density arrest (unpublished observation). As has been shown by other workers (4, 41), heterogeneity in expression was also observed for several other surface differentiation markers expressed on *Theileria*-infected cells (Table 1).

Clevers et al. reported that the surface marker WC1 is expressed only on $CD4^-$ $CD8^-$ T lymphocytes expressing the γδ T-cell receptor (15). Baldwin et al., however, have shown that a proportion of cells within some cloned *T. parva*-transformed B-cell lines expressed WC1 (4). In the present work, we show that *T. parva*-infected T cells expressing CD2, CD4, or CD8 can also express WC1. It is not clear whether the parasite is directly involved in inducing WC1 expression or whether the WC1 gene is spontaneously derepressed in culture.

In all cell lines tested except the newly infected IN cell lines, virtually 100% of the cells expressed MHC class II molecules. In the cell lines IN3, IN7, IN9, and IN10, a proportion of the cells were negative for MHC class II expression. The "cycling expression" of MHC class II molecules on the surface of *Theileria*-infected cells has been documented before (P. A. Lalor, Ph.D. thesis, Brunel University, Uxbridge, U.K., 1983). It will be interesting to see whether the IN cell lines, with time in culture, will also progress to >99% expression.

The expression of IL-2R on a *T. annulata*-infected cell line has recently been demonstrated by biochemical and affinity-labeling studies (28). This IL-2R consisted predominantly of the intermediate-affinity β-chain, but small amounts of α-chain was also detected. The three *T. annulata*-infected cell lines that were analyzed here clearly showed Tac antigen expression, and their proliferation was enhanced by rIL-2. Spooner et al. (53, 54) have recently shown that *T. annulata* preferentially infects MHC class II-positive cells but does not infect T cells. Whereas B cells can be infected and transformed by both parasites (1, 53, 54), albeit more readily by *T. annulata* (53, 54), monocytes are transformed only by *T. annulata*. These authors also reported that *T. annulata*-infected cells fail to express the differentiation markers CD4 and CD8. The fact that all Ta(Ä)-infected cells expressed surface IgG suggests that they are of B-cell origin. Our experiments, however, show that all three *T. annulata*-infected cell lines tested expressed CD2 and one expressed CD4. This finding agrees with the finding of Ahmed et al., who have shown that bovine mononuclear cells expressing the sheep erythrocyte receptor (= CD2, also called T11) could be transformed by infection with *T. annulata* (1). Since the expression of CD2 or CD4 markers has been reported to be characteristic of bovine T cells (6, 41), *T. annulata*-infected cells could be considered to be of T-cell lineage. The expression of these markers may, on the other hand, not be entirely restricted to T cells. Sheep macrophages, for instance, have been shown to express CD2 molecules (W. R. Hein, personal communication), and CD4 expression on human monocytes has also been described (37). Furthermore, the fact that *T. annulata*-infected cells were negative for CD3γ mRNA expression also argues strongly against their being T cells.

Tac antigen expression by gamma interferon-treated human leukemic or normal monocytes has been described (26, 29). There are varying reports on the functional role of IL-2R expression on monocytes versus T or B cells. Contrary to observations made for activated T or B cells, Holter et al. (29) failed to find evidence of IL-2-induced proliferation in monocytes. Instead, IL-2 increased lipopolysaccharide-induced IL-1β secretion of gamma interferon-treated monocytes. In contrast, Baccarini et al. (3) have shown that murine macrophage precursors stimulated by relatively high concentrations of IL-2 were able to proliferate and differentiate to macrophages in the absence of any other factors. In this case, however, the Tac component of the IL-2R did not appear to be involved, since it could not be detected on the surface of these cells. We have shown that *T. annulata*-infected cells expressing Tac antigen respond to rIL-2 by enhanced proliferation. The importance of Tac antigen expression may differ for *T. parva*- and *T. annulata*-infected cells. This is suggested by the fact that the Ta(Ä) cells could proliferate even though only few cells expressed IL-2R.

Our original proposal, that the constitutive expression of functional IL-2R on *T. parva*-infected cells may be an important factor in the growth of parasitized lymphocytes in vitro (16, 17), is strengthened by several new observations. rIL-2 improves both the cloning efficiency of *T. parva*-infected cells and the transformation rate in vitro (unpublished data). This is also supported by the work of Brown et al. (11), in which *Theileria*-infected cell lines were treated with parvaquone, a compound related to but less effective than BW720c. Contrary to BW720c, treatment with parvaquone does not always result in the complete elimination of viable parasites from the infected cells. The recovery and proliferation of *Theileria*-infected cells from parvaquone-treated cultures was once again dependent on the addition of rIL-2, demonstrating a role for exogenous IL-2 in promoting the growth of parasite-infected cells when they constitute a minority in culture.

It has been reported previously that infection with *T. parva* results in autocrine growth, involving the secretion of a growth factor with IL-2-like activity. We have recently obtained evidence that the IL-2 gene is transcribed, albeit at very low levels, in most of the *T. parva*-infected cell lines

tested (V. Heussler and D. A. E. Dobbelaere, unpublished observation). The continuous proliferation of *T. parva*-infected cells in the infected host may therefore be driven in part by an autocrine IL-2–IL-2R loop. *T. parva*-infected cells also cause polyclonal stimulation of autologous uninfected cells, a phenomenon referred to as the autologous *Theileria* mixed leukocyte reaction (22, 24, 44, 45). IL-2 secreted by polyclonally activated uninfected cells can thus contribute directly to the proliferation of IL-2R-expressing parasitized cells and also indirectly by inducing increased IL-2R/Tac gene expression. Since the schizont constitutes the pathogenic stage of the parasite and has been shown to multiply in synchrony with the proliferating lymphocyte, it can be concluded that paracrine as well as autocrine stimulation of *T. parva*-infected cell growth will contribute to the pathogenesis of East Coast fever.

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