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Monoclonal Antibodies Prepared against *Theileria annulata*-infected Bovine Cells Recognize an Infection-associated Antigen Expressed by the Infected Cells

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SUMMARY

A battery of monoclonal antibodies were produced in mice against *Theileria annulata*-infected cells. Two of these antibodies (IgM and IgG1) reacted specifically with the surface membrane of the infected cells but not with Con A-stimulated or unstimulated bovine peripheral blood lymphocytes (PBL). The antibodies could stain also clones which were derived from the cell lines tested in the present study. Similarly, they reacted with *T.annulata*-infected but not with uninfected sheep PBL. After removal of the parasites by treating the infected cells with the anti-theilerial drug buparvaquone, the antibodies were no more able to bind to the cells.

INTRODUCTION

Theileria annulata, the causative agent of tropical theileriosis, induces a severe lymphoproliterative disease in cattle. Sporozoites of *Theileria* species invade the host cells, where they subsequently differentiate to macro- and microschizonts (Neitz 1957). Different cell types, including T and B cells, and monocytes/macrophages are infected, transformed and induced to a permanent proliferation by *T. annulata* (Ahmed et al., 1984; Spooner et al., 1989; Glass et al., 1989). Co-cultivation of these infected cells and uninfected autologous lymphocytes initiates proliferative responses in the uninfected cell population (Pearson et al., 1979; Emery and Morrison, 1980; Ahmed et al., 1981).
During infection with *Theileria annulata* and *T. parva*, a subset of T cells are activated which are capable to lyse specifically parasitized autologous cell lines (Eugui and Emery, 1981; Emery et al., 1981; Preston et al., 1983; Ahmed et al., 1989; Innes et al., 1989). This has been suggested to be due to the expression of a neo-antigenic determinant by the infected cells. However, there is no direct evidence for the existence of such an antigen, since no antibody activity against the surface membrane of the infected cells has been demonstrated in serum of *Theileria*-infected or immune cattle (Duffus et al., 1978; Ahmed et al., 1988).

Monoclonal antibodies have been produced against *T. annulata* (Shiels et al., 1986) and *T. parva*-infected cells (Newson et al., 1986). Although these antibodies allowed the demonstration of a neo-antigenic determinant on the surface membrane of the infected cells, a direct evidence that this antigen is coded by the parasites is still missing. In the present study, we produced two monoclonal antibodies, IgM and IgG1, which are able to react with *T. annulata*-infected cells, but not with uninfected cells or with those cells from which the schizonts were removed by chemotherapy. Evidence is presented for a causal relation between the infection of the cells with the parasites and the expression of the neo-antigenic determinant.

MATERIALS AND METHODS

Preparation of bovine peripheral blood lymphocytes.

Bovine peripheral blood lymphocytes (PBL) were prepared by density gradient centrifugation on Ficoll-Paque, using the method of BOyum (1968). After centrifugation at 400 g for 30 min, the mononuclear cells were collected from the interphase, washed in RPMI-medium (RPMI 1640) containing 100 μg/ml penicillin/streptomycin, 10% heat-inactivated foetal calf serum (FCS), and 2 mM L-glutaraine (Biochrom, Berlin).

Establishment of *T. annulata*-infected cell lines

Sporozoites of *T. annulata* (the Ankara-strain) were prepared from salivary glands of infected *Hyalomma anatolicum excavatum* ticks and used for preparation of *T. annulata*-infected cell lines (macroschizont-containing cell lines) as described by Brown et al. (1973). For in vitro infection, 0.1 ml of the sporozoite suspension was added to 1 x lO^7 PBL/ml in 13-ml plastic tubes. The cells were then incubated at 37^OC in air with 5% CO_2. The medium was renewed daily up to 7 days, then twice a week.

Cloning of *T. annulata*-infected cells

Cells were cloned by limiting dilution technique into 96 well flat bottomed microtiter plates, into which $6xl0^3$ mouse peritoneal cells were previously added, using RPMI-medium containing the above mentioned supplements to give 0.3 cells per well. The growing clones were then expanded and aliquotes cryopreserved at 180 ^OC.

Treatment of *T. annulata*-infected cells with buparvaquone

The infected cells were adjusted to 5 x $l0^4$/ml and incubated in tissue culture flasks with 0.05 /μg buparvaquone (Butalex^R, Coopers Animal Health) at 37^OC for different days. Thereafter, the cells were collected, washed and adjusted to appropriate concentrations (Rintelen et al., 1990).

Preparation of Con A-blasts

Con A-blasts were prepared by stimulating 1 x 10^6 PBL/ml with 4 μg Con A. The cells were cultured for 72 h in medium containing the above mentioned substances.

Preparation of monoclonal antibodies

For production of monoclonal antibodies Balb/c mice were intraperitoneally immunized by inoculation of 1.5 x 10^7 *T. annulata*-infected cells (the Ankara-strain). After 11, 22 and 33 days, the mice were boostered with the same dose of the infected cells. Four days after the last immunization their spleen cells were prepared and fused with NS1 cells. The hybridoma cells were selected in medium containing hypoxanthine/aminopterin/thymidine (HAT). Antibody producing hybrids were detected using the indirect immunofluorescence test (IFAT).

Indirect immunofluorescence test

The supernatants of the hybrids were tested for their antibody activity by adding them to viable *T. annulata*-infected cells which were previously adhered to special slides (Bio-Rad Adhesion Slides, Bio-Rad, München). The slides were kept at 4 ^{O}C for 1 h, then washed twice in PBS, fixed for 10 min with ice cold 3% formalin. Thereafter, biotinelated sheep anti-mouse Ig was added to each well and incubated for 45 min at 37 ^{O}C. After two washes fluoresceine-streptavidin was added, further incubated at 37 ^{O}C for 30 min, washed twice and then counterstained with 0.01% Evans blue, mounted with 50% glycerol in PBS and examined by fluorescence microscopy.

RESULTS

Binding of the monoclonal antibodies to *T. annulata*-infected bovine cells.

Monoclonal antibodies against *T. annulata*-infected bovine cells were produced in mice and their specificity was screened using IFAT. Two of these antibodies could stain the surface membrane of all *T. annulata*-infected cell lines tested in the present study. Clones which were prepared from the cell lines used for the immunization of the mice or from other cell lines could also be stained by these antibodies. However, none of these antibodies was able to react with normal or Con A-stimulated PBL (Tab. l). This would mean that the antibodies recognize an infection associated-antigen.

Tab.l: Binding of the antibodies to *T. annulata*-infected and uninfected bovine cells.
+ = positive reaction; - = no reaction

Cells tested	monoclonal antibodies	
	D6D6	G5E7
PBL 346	-	-
Con A-blasts 346	-	-
Infected cell line 346	+	+
clones from cell line 346: 1D9	+	+
2D5	+	+
3D7	+	+
1F4	+	+

Binding of the monoclonal antibodies to *T. annulata*-infected sheep cells.

In a previous paper, we have shown that *T. annulata* can transform bovine as well as sheep lymphocytes in vitro (Steuber et al., 1986). Therefore, we attempted to examine whether the monoclonal antibodies, which were produced against macroschizont-containing bovine cells, could also react with cell lines prepared from sheep PBL. As control, we used Con A-stimulated and unstimulated sheep PBL. Again only *T. annulata*-infected cells were stained by the antibodies (Tab.2). This suggests that sheep cells, after being infected by *T. annulata* in vitro, express a new antigenic determinant(s) which resembels that expressed by bovine cells infected with the same parasite.

Tab.2: Binding of the antibodies to *T. annulata*-infected and uninfected sheep cells.
+ = positive reaction; - = no reaction

Cells tested	monoclonal antibodies	
	D6D6	G5E7
T.annulata-infected sheep cell line 1	+	+
PBL	-	-
Con A-blasts	-	-

Removal of the parasites by buparvaquone prevents the binding of the monoclonal antibodies to the cells.

If the cells express an infection-associated antigen, then the elimination of the parasites must lead to the inhibition of the expression of the supposed antigen. For this reason, the infected cells were treated with buparvaquone, a drug which can effectively eliminate the intracellular schizonts. Microscopical examination showed that buparvaquone indeed removed the schizonts from the cells after 8 days of treatment (data not shown). Such cells were then incubated with the monoclonal antibodies. In contrast to untreated cells (Fig.1), bovine cells from which the parasites were eliminated could not react with the antibodies (Fig.2). Similarly the antibodies did not bind to sheep cells after removal of the parasites (data not showed).

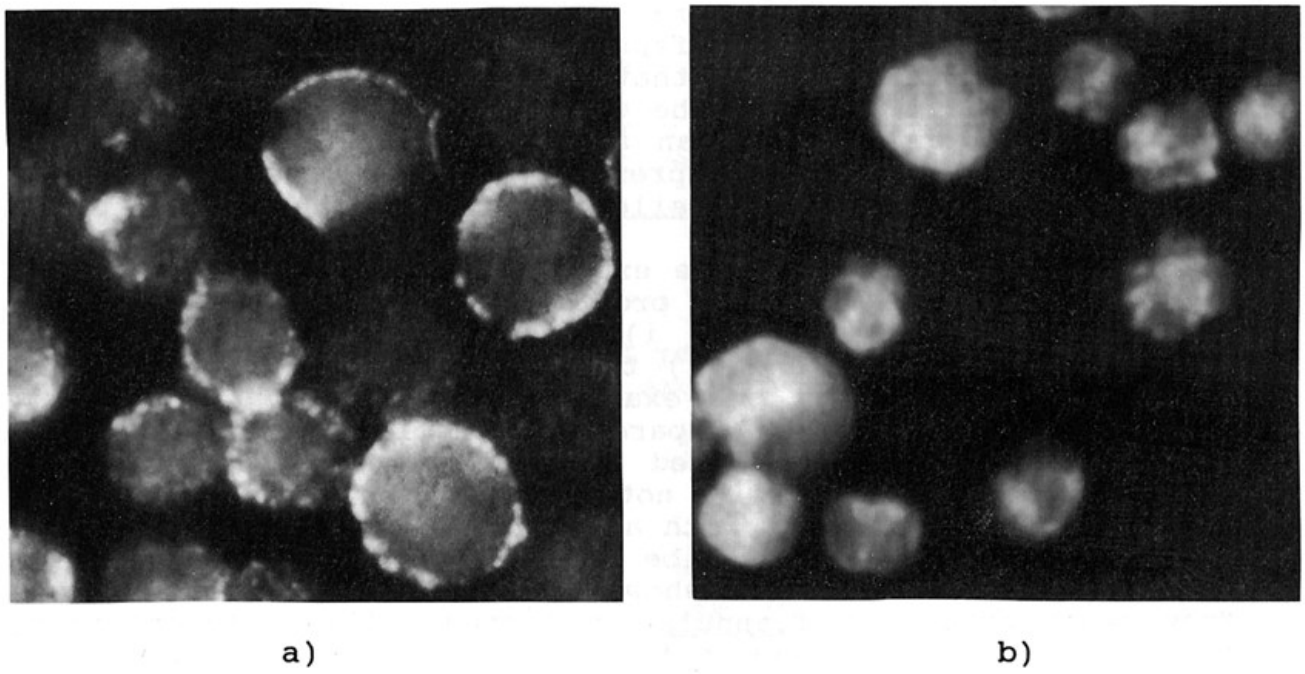

Fig.1. Binding of D6D6 to *T. annulata*-infected cells, (a) before and (b) after treatment with buparvaquone

Determination of the isotype of the monoclonal antibodies

In order to determine the isotype of the antibodies we used a mouse monoclonal antibody isotyping kit (Amersham Buchler GMBH &Co KG, Germany). The results obtained showed that one of the antibodies (G5E7) was of IgM and the another one (D6D6) of IgG1 isotype.

DISCUSSION

One of the most important characteristics of theileriosis is the highly proliferative reaction of the schizont-infected cells. Beside this, the infected cells have been shown to be strong stimulators in a mixed lymphocyte culture (Pearson et al., 1979; Pinder et al., 1981; Ahmed et al.,1981). Both these properties are presumed to be induced by the intracellular parasites (Pearson et al., 1979; Ahmed et al., 1988). In a

recent paper, Rintelen et al. (1990) presented evidence that the elimination of the parasites by buparvaquone leads to a complete inhibition of the generation of the mixed lymphocyte reaction. This would mean that the antigens which are expressed after transformation of the cells are no more expressed after the removal of the schizonts. Despite these facts, no direct evidence has been presented for the existence of such a parasite-induced neo-antigenic determinant. Thus the only evidence is based on two findings:

a) The capacity of the infected cells to stimulate autologous uninfected lymphocytes. However, some authors believe that such a reaction is unspecific and is thought to be due to a mitogenic effect of jet undetermined antigenic determinants (Pinder et al., 1981; Goddeeris and Morrison, 1987).

b) The strain specificity of *T. parva*-immune cells. These cells have been shown to kill only those target cells which are infected with the same strain of the parasite that have been used for the immunization of the animals from which the immune cells are derived. This finding suggests that a new antigenic determinant(s) has induced the activation of the cytotoxic killer cells. However, attempts to characterize such an antigen have failed, because no antibody activity against the presumed antigenic determinant has been found in the serum of *theileria*-infected cattle (Duffus et al., 1978; Ahmed et al., 1988).

These findings indicate that the expressed antigen can probably be recognized only by T-cells. In order to identify these antigens, it is necessary to prepare i) T-cell clones specific for *theileria*-infected cells and ii) to produce monoclonal antibodies against the infected cells, for example in mice.

Shiels et al. (1986) have prepared a monoclonal antibody which reacted with *T. annulata*-infected cells. However, the parasite-specificity of this antibody is not clear and it reacted only with a certain population but not with all infected cells.

In the present study, we describe two monoclonal antibodies which have been produced in mice and show the following properties:

a) They react only with *T. annulata*-infected cells. Interestingly, they could react with sheep cells also provided the cells were infected with schizonts of *T. annulata* in vitro. This provided evidence that the antibodies did not bind to *T. annulata*-infected cells via BoLA-antigens.

b) Neither normal nor Con A-stimulated cells could react with the antibodies. This indicates that the expression of the antigen is associated with the infection of the cells and not with differentiation antigens which appear on transformed cells.

c) After removal of the parasites by buparvaquone, the cells were no more able to interact with the antibodies, suggesting the importance of the intracellular presence of the schizonts. This would mean that the schizonts induced the expression of the new antigenic determinants on the surface of the parasitized cells. The presumed molecules disappear after removal of the parasites by buparvaquone.

These molecules are probably the same structure which stimulate uninfected PBL in an autologous mixed lymphocyte culture to DNA-synthesis. It is interesting to note that the capacity of the infected cells to stimulate uninfected autologous lymphocytes (Rintelen et al., 1990) and, as described in the

present study, to interact with the monoclonal antibodies disappear after removal of the schizonts by buparvaquone. This would mean that the antibodies described in the present paper probably recognize those molecules which induced the proliferation of the uninfected autologous cells.
In contrast to *T. parva, T. annulata* can infect also sheep cells (Steuber et al., 1987), indicating that *T. annulata* has a wider host range. In the present paper we describe for the first time that monoclonal antibodies which have been produced against *T. annulata*-infected bovine cells in mice can also recognize antigens expressed by sheep lymphocytes infected with *T. annulata*. This indicates that the antigen processing and its expression in *T. annulata*-infected bovine and sheep cells functions in a similar way and is not subjected to modifications due differences in the animal species.

Two further unpublished observations suggest that the antibodies may bind to the same target molecule through which the cytotoxic cells recognize their targets. Thus, they could inhibit the lytic effect of cytotoxic cells on the *T. annulata*-infected cells and suppress the generation of an autologous mixed lymphocyte reaction between infected and uninfected cells in vitro. Therefore, we believe that they may be usefull tools for the characterization and preparation of those antigenic determinants which are relevant for the induction of a protective cell mediated immune response against *T. annulata* infection.

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