Antibodies against Major Histocompatibility Complex Class II Antigens Directly Inhibit the Growth of T Cells Infected with *Theileria parva* without Affecting Their State of Activation

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Summary

We have analyzed the effect of antibodies (Abs) directed against major histocompatibility complex (MHC) class II Abs on the proliferation of *Theileria parva*-infected (Tpi) T cells. Anti-MHC class II Abs exert a direct effect on Tpi T cells causing an acute block in their proliferation. The inhibition does not involve apoptosis and is also entirely reversible. The rapid arrest of DNA synthesis caused by anti-MHC class II Abs is not due to interference with the state of activation of the T cells since the transcriptional activator NF-κB remains activated in arrested cells. In addition, interleukin 2 (IL-2), IL-2R, and c-myc gene expression are also unaffected. By analyzing the cell-cycle phase distribution of inhibited cells, it could be shown that cells in all phases of the cell cycle are inhibited. The signal transduction pathway that results in inhibition was shown to be independent of protein kinase C and extracellular Ca2+. Tyrosine kinase inhibitors, however, partly reduced the level of inhibition and, conversely, phosphatase inhibitors enhanced it. The possible relevance of this phenomenon in other systems is discussed.

T cells that become infected with the protozoan parasite *Theileria parva* lose their dependence on antigen-specific priming and acquire the ability to proliferate continuously in vitro. Like normal T cells, however, they remain dependent for their proliferation on the expression of the IL-2 and IL-2R genes (1, 2). One significant difference between *T. parva*-infected (Tpi)† T cells and permanently transformed T cell lines is the lymphoblastoid transformation caused by infection with the parasite is completely reversible. T cells return to a resting phenotype after elimination of the parasite using the parasite-specific drug BW720c (3). The way in which the parasite induces continuous proliferation is not entirely understood, but it has been shown in a number of Tpi cell lines that the continuous proliferation involves an IL-2/IL-2R–mediated autocrine loop that is strictly dependent on the presence of the parasite in the T cell cytoplasm. Cells from which the parasite has been eliminated by drug treatment can be maintained in culture for several more weeks, but only in the presence of exogenously added IL-2 and regular stimulation with phorbol esters. Because of the reversibility of their transformation, Tpi T cells provide an excellent model for the dissection of the various mechanisms that control T cell proliferation. In the present work, we examined the effects of anti-MHC class II Abs on the proliferation of Tpi T cells.

Class II antigens of the MHC play a key role in immune responses and are best known for their role in antigen presentation. However, additional, regulatory roles associated with monomorphic and polymorphic determinants of these molecules have recently been identified. It has been shown that MHC class II molecules expressed on lymphocytes are capable of transducing signals (4–10), and there are several reports on the biological effects of anti-MHC class II Abs on monocytes (11, 12) and B cells (4, 5, 13–16). In this regard, anti-MHC class II Abs have been shown to be potent inhibitors of B cell proliferation (10, 11, 14, 15). Activated T cells also express MHC class II molecules, but the biological consequences of the engagement by Abs of MHC class II molecules expressed on T cells is less clear than for B cells. Previous in vitro studies on T cells, stimulated to proliferate using anti-CD3 Abs in the presence of monocytes, have shown that the inhibition of T cell proliferation by anti-MHC class II Abs is predominantly effected indirectly through interference with the T cell activation pathway (12, 17–19). Anti-MHC class II Abs were shown to exert a direct effect on...
the monocytosis and, as a consequence, IL-2 and IL-2R, gene expression by T cells was reduced significantly. A limited direct effect on T cell proliferation could also be demonstrated (12). Mixed cultures of T cells and monocytosis, however, do not allow the indirect and direct effects of anti-MHC class II Abs on T cells to be distinguished easily. Cell lines, consisting of continuously proliferating transformed cells such as lymphoma cells, are often used to address such problems. Permanently transformed cells of T cell lineage often possess altered genomes, however, and in many cases lose growth factor dependence. For these reasons, they may not always be representative of normal activated T cells. Tpi T cells fulfill this requirement more closely and, in this work, we describe how anti-MHC class II Abs can directly inhibit T cell proliferation in a reversible manner.

Materials and Methods

**Cells.** The characteristics and maintenance of Tpi T cell clones have been described previously (20). The Tpi T cells used in this study were TpM(803) cells that are of the CD4⁺CD8⁻ phenotype and express the TCR-α/β. Cells were cultured at 37°C in Dulbecco's L15 medium containing 20 mM Hepes (pH 7.1), benzylpenicillin (100 U/ml), streptomycin (100 μg/ml), l-glutamine (20 μg/ml), 10% (vol/vol) heat-inactivated fetal bovine serum (all from Gibco Laboratories, Basel, Switzerland), and 50 μM 2-ME. Cell cultures were passaged every 2-3 d and their density was usually 1-8 x 10⁵/ml. To eliminate the parasite from the T cells, the theilericidal drug BW720c (21) was added to the culture medium at a concentration of 50 ng/ml as described before (3). Recombinant IL-2 used in a number of experiments was purchased from Amersham (Braunschweig, FRG; [ala125]interleukin 2, code ARN 7010).

**Anti-MHC Class II Inhibition Experiments.** To test the antiproliferative activity of anti-MHC class II Abs, Tpi T cells (10⁴ cells/well in 200 μl) were cultured in medium containing 5% (vol/vol) of dialyzed anti-MHC class II hybridoma supernatant (corresponding to 7.5 μg/ml of Ig; anti-HLA-DR; Code Nr. M 7004; Dakopatts, Hamburg, FRG) or the equivalent amount of control Ab. The anti-MHC class II Abs, which bind to monomorphic regions of the bovine MHC class II molecules, and the control Abs (UPC 10, Code Nr. M-9144; Sigma, München, FRG) were of the IgG₂a isotype. Both Ab preparations were dialyzed extensively against Dulbecco's L15 medium which was used for the culture of Tpi T cells, and the FCS concentration was adjusted to that of the Tpi T cell culture medium (10% vol/vol). Preliminary experiments confirmed that inhibitory activity was contained in the protein A-binding fraction and unspecific activity of the hybridoma supernatant was excluded by testing it on a variety of different cell lines. Proliferation was monitored by measuring the incorporation of T cells grown in the presence or absence of the inhibitor were then compared and are presented (see Fig. 4).

**Transcription and Transfection Analysis.** Tpi T cells were transfected with the plasmid construct -121/-232 HIV-CAT which contains two NF-κB binding sites that regulate its expression. Details on the construct and transfection assays on Tpi T cells were reported before (23). A total of 5 x 10⁴ cells were used per assay. After transfection, cells were cultured in normal medium or medium containing anti-MHC class II or control Abs. Lysates were prepared after 24 and 48 h and tested for chloramphenicol acetyl transferase (CAT) activity. The preparation of nuclear extracts and gel retardation analysis were performed as described previously (24); 5 x 10⁴ cells were used per assay. The oligonucleotide used for gel retardation assays contains the NF-κB binding sequence motif which is also present in the HIV-LTR.

**Northern Blot and PCR Analysis.** The Tpi T cells were cultured in 25 cm² flasks in the absence or presence of anti-MHC class II Abs. Northern blot analysis of polyadenylated RNA using the bovine IL-2R/Tac and actin cDNA probes was performed as described before (20, 22, 25). The bovine c-myc probe originates from a genomic clone and contains exon 1- and exon 2–specific sequences (Dobbelare, D.A.E., unpublished results). Control B cell mRNA was prepared from the bovine B cell line B155 (donated by Dr. L. Droogmans, Université Libre de Bruxelles, Brussels, Belgium). PCR analysis of IL-2 gene transcription using IL-2–specific primers was performed as described (1). Control PCR using actin–specific primers confirmed the quality of the RNA and cDNA synthesis of the various RNA preparations (data not shown).

**Cell Cycle Analysis.** Tpi T cells were cultured in 96-well plates in 200 μl of medium containing either control or anti-MHC class II Ab. At the times indicated, cells were harvested from wells of wells and briefly trypsinized to disperse clumps. To avoid extensive cell lysis during ethanol fixation, cells were fixed (0–30 min) in 37°C 270 μl 100 μg/ml PI in PBS were added and cells stored in darkness at 4°C for at least 6 h before analysis. Measurements were made on a FACStar Plus®/Consort 30 system (Becton Dickinson, Heidelberg, FRG) using PBS sheath fluid and 150 mW of excitation light at 488 nm. Red (PI) fluorescence was collected through a DF630/38 filter. List mode data was processed with the Cellfit program (Becton Dickinson) using computer gating on fluorescence pulse area, height, and width to generate histograms only of intact single cells with an apparent DNA content between 2 and 4 times the haploid complement. These were analyzed by the software's sum of broadened rectangles (SOBR) model to give a goodness-of-fit (χ²) of between 1.06 and 1.40 in the experiment shown.

**Tyrrosine Kinase and Phosphate Inhibitor Studies.** Before the addition of Abs to the culture medium, cells were cultured overnight in the presence of either 1 μM herbimycin A, 1 μg/ml genistein (both from Calbiochem, Bad Soden, FRG) or 15 μM sodium orthovanadate (Sigma). Cells were then washed twice in medium and cultured (10⁴ cells/well in 200 μl) in the presence or absence of anti-MHC class II Abs in normal medium or medium containing one of the following inhibitors: herbimycin A (1 μM), genistein (10 μg/ml), or sodium orthovanadate (15 μM). The percent inhibition induced by treatment with anti-MHC class II Abs obtained for cells grown in the presence or absence of the inhibitor were then compared and are presented (see Fig. 4).

**Results**

**Anti-MHC Class II Abs Cause the Rapid Arrest of Tpi T Cell Proliferation.** Like activated T cells of most species, Tpi T cells express MHC class II molecules on their surface (20). When anti-MHC class II Abs were added to the culture medium of Tpi T cells a rapid arrest of their proliferation
could be observed. Fig. 1A shows an experiment in which DNA synthesis of Tpi T cells, measured by [3H]Tdr incorporation, was reduced to <50% within 4 h and >90% inhibition was reached by 24 h. The inhibition at 24 h observed in most experiments could range from 70 to >95%. Inhibition did not result from apoptosis, since DNA fragmentation or changes in cell viability could not be observed. In addition, the inhibition proved to be reversible, since DNA synthesis resumed upon removal of the Abs from the culture medium, and cells achieved a proliferation rate comparable with that of control cultures within 24 h (Fig. 1B).

It has been shown that the inhibition of anti-CD3-stimulated T cells by Abs directed against MHC class II molecules in the presence of monocytes (12) could be abrogated by the addition of IL-2 to the culture medium. In contrast to these observations, the addition of rIL-2 to the culture medium of Tpi T cells inhibited by anti-MHC class II could not restore proliferation (Fig. 1C). Inhibition was not restricted to this particular CD4+CD8− Tpi T cell line since other, CD4−CD8+ and CD4−CD8− Tpi T cells were also inhibited (data not shown). Inhibition was also not dependent on the T cells being infected with T. parva, since T cell clones that were cured of the parasite, but cultured under conditions which allow them to continue to proliferate (3) were also inhibited. Furthermore, the addition of rIL-2 again failed to abrogate the inhibition of proliferation mediated by the anti-MHC class II Abs (Fig. 1D).

The Activation of the Transcription Factor NF-κB Is Not Inhibited. One of the typical features of early T cell activation is the activation of the transcription factor NF-κB which is involved in the regulation of IL-2 (26) and IL-2R gene expression (27). In Tpi T cells, NF-κB can also be found in the activated form in the nucleus (23). Since it had been proposed that anti-MHC class II Abs cause the downregulation of T cell activation and consequently of IL-2 and IL-2R expression (12, 17, 18), we examined the effect of these Abs on the activation of NF-κB. Band shift analysis using nuclear extracts from control Tpi T cells or cells treated with anti-MHC class II Abs revealed no difference in the level of NF-κB activity (Fig. 2A). To test if NF-κB was also biologically functional, Tpi T cells treated with anti-MHC class II were transfected with the plasmid HIV-CAT in which CAT gene expression is controlled by the two NF-κB binding sequences contained in the HIV LTR. No difference in CAT activity could be observed when cells treated with anti-MHC class II were compared with control cells (Fig. 2B).

Anti-MHC Class II Abs Do Not Inhibit IL-2, IL-2R, and c-myc Expression. It has been reported for T cells stimulated to proliferate by anti-CD3 Abs in the presence of monocytes, that the addition of anti-MHC class II Abs to the culture medium results in a reduction of IL-2R and IL-2 expression (18). Northern blot analysis showed that treatment of Tpi T cells with anti-MHC class II Abs did not inhibit IL-2Rα chain (p55) mRNA expression, which is normally induced upon T cell activation and also in Tpi T cells (Fig. 3A). Likewise, the expression of the proto-oncogene c-myc, normally induced upon T cell activation, was not inhibited. Analysis by PCR (Fig. 3B) revealed that IL-2 mRNA expression is also not inhibited by anti-MHC class II Abs, whereas, in a control experiment, elimination of the parasite by addition of the theileridial drug BW720c clearly resulted in the disappearance of IL-2 mRNA.
Figure 2. Analysis of the transcriptional activator NF-κB in anti-MHC class II-treated Tpi T cells. (A) Gel retardation analysis with 32P-labeled NF-κB-binding oligonucleotides. Binding assays were performed using nuclear extracts prepared from control Tpi T cells or Tpi T cells cultured for 24 h in the presence of anti-MHC class II or control Abs. (B) Demonstration of NF-κB activity by HIV-CAT transfection assay. Tpi T cells were transfected with the plasmid construct (–121/232) HIV-CAT which depends on the presence of activated NF-κB for its expression. After transfection, cells were cultured in normal medium or medium containing anti-MHC class II or control Abs. Lysates were prepared after 24 and 48 h and tested for CAT activity.

These data and the experiments carried out on the activation of NF-κB strongly suggest that the inhibition of proliferating Tpi T cells by anti-MHC class II Abs is not due to an inhibition of the IL-2/IL-2R activation pathway.

Cells Are Inhibited in All Stages of the Cell Cycle. Experiments were also carried out to determine in which phase of the cell cycle cells are arrested after treatment with anti-MHC class II Abs. Cells were incubated with anti-MHC class II Abs or control Abs and at different times, ranging from 3 to 50 h after Ab addition, proliferation was monitored by measuring [3H]TdR incorporation. Cell-cycle distributions were analyzed in parallel by carrying out PI staining of the DNA followed by flow cytometry. Surprisingly, no substantial cell-cycle phase differences could be detected between arrested cells and control cells, even in experiments where [3H]TdR incorporation was inhibited up to 99%. A representative experiment is shown in Table 1. These results indicate that cells that are inhibited in their proliferation by anti-class II Abs do not gather in a defined stage of the cell cycle, but, instead, are arrested in all stages of the cell cycle.

Anti-MHC Class II–mediated Inhibition Is Independent of Protein Kinase C (PKC) and Extracellular Ca++/ but Involves Tyrosine Phosphorylation. MHC class II–mediated signals have been shown to result in the mobilization of Ca++ changes in the protein tyrosine phosphorylation patterns (3, 6), and the activation of serine/threonine-specific PKC (7). One of the consequences of ligand engagement of MHC class II molecules is the expression of lymphocyte adhesion molecules, resulting in pronounced lymphocyte aggregation (28). It has been shown that the induction of adhesion involves the activation of PKC and is sensitive to inhibitors of PKC (16). Treatment of Tpi T cells with anti-MHC class II Abs also induced extensive aggregation of Tpi T cells that could be inhibited by the PKC inhibitor H7 and the chelating agent EGTA. The inhibition of DNA synthesis caused by treatment with anti-class II Abs, however, was not affected by either reagent (data not shown). Treatment of the cells with the tyrosine kinase inhibitors genistein and herbimycin A, on the other hand, partly abrogated the inhibition, whereas the phosphatase inhibitor

Figure 3. IL-2R/Tac, c-myc, and IL-2 mRNA expression in Tpi T cells treated with anti-MHC class II Abs. (A) Northern blot analysis of IL-2R/Tac and c-myc mRNA expression in Tpi T cells cultured in the presence or absence of anti-MHC class II Abs. Filters were rehybridized with a bovine cDNA actin probe as a control for the amount of RNA loaded. (B) PCR analysis of IL-2 mRNA expression in Tpi T cells cultured in the presence of control Abs, anti-MHC class II Abs, or in culture medium without additives. A control PCR demonstrating the disappearance of IL-2 mRNA transcripts upon elimination of the parasite was performed on mRNA isolated from cells cultured for 3 d in the presence of the theliferidial drug BW720c. Polyadenylated tRNA isolated from a bovine B cell line was used as an additional negative control.

772 Inhibition of T Cell Proliferation by Anti-MHC Class II Antibodies
Table 1. Cell-cycle Distribution Analysis of Tpi T Cells Arrested in Their Proliferation by Treatment with Anti-MHC Class II Abs

| Time | Antibody | Percent cells in (Percent) | Percent [H]Tdr incorporation (± SD) |
|------|----------|---------------------------|-----------------------------------|
|      |          | G1       | S        | G2 + M    |                               |
| 0    | None     | 27.6     | 58.8     | 13.6      | ND                               |
| 24   | Control Ab | 29.6     | 56.5     | 13.9      | 100.0 (± 8.0)                    |
|      | Anti-class II | 28.4     | 58.0     | 13.6      | 28.5 (± 1.5)                     |
| 50   | Control Ab | 27.5     | 62.7     | 9.8       | 100.0 (± 15.0)                   |
|      | Anti-class II | 29.8     | 59.8     | 11.5      | 0.9 (± 0.1)                      |

Tpi T cells were cultured in normal medium in the presence or absence of control or anti-MHC class II Abs. At the times indicated, cells were harvested, fixed in 1% paraformaldehyde/0.5% saponin and DNA staining carried out with PI. Cells were then analyzed on the FACS® for relative DNA content. [H]Tdr incorporation was measured in parallel and is expressed as a percentage of the value obtained for cells treated with control Ab.

sodium orthovanadate was capable of enhancing the inhibitory effects caused by anti-MHC class II treatment (Fig. 4) suggesting the involvement of tyrosine kinase activity in the signal cascade resulting in the arrest of proliferation.

Discussion

In the present paper, we show that the engagement of MHC class II molecules, which are expressed on Tpi T cells, can result directly in the inhibition of their growth. This negative regulation is unusual in that it involves a rapid arrest of DNA synthesis upon treatment with anti-MHC class II Abs, without affecting the state of activation of the T cell, as measured by NF-κB activity and IL-2 and IL2-R gene expression. In addition, the inhibition does not involve apoptosis and is totally reversible.

Figure 4. The effect of phosphatase and tyrosine kinase inhibitors on anti-MHC class II-mediated inhibition of Tpi T cells. Cells were first cultured overnight in the presence of the various inhibitors and then plated out and cultured for a further 24 h in medium containing the inhibitors in the presence or absence of anti-MHC class II Abs. Proliferation was monitored by measuring [H]Tdr incorporation. (Black bar) Control cultures. (Open and gray bar) Percent proliferation compared with control cultures for cells treated with anti-MHC class II Abs in the absence or presence of the inhibitor.

Our observations differ substantially from earlier work describing the inhibitory effect of anti-MHC class II Abs on T cells stimulated to proliferate by anti-CD3 Abs in the presence of monocytes (12, 18, 19), or in MLCs (17). In these previous studies it was shown that the monocytes are the main target of inhibition and that the arrest of T cell proliferation occurred through blocking their activation. Inhibition was due to interference with cell–cell interactions between macrophages and T cells at the early phases of activation (12, 19), and negative effects on IL-1β and IL-6 mRNA expression were also reported (18). In the same studies, IL-2 and IL-2R expression in the affected T cells were also clearly reduced. In Tpi cells arrested by anti-MHC class II Abs, no decrease in IL-2, IL-2R, and c-myc mRNA expression could be detected, and the transcriptional activator NF-κB, which is involved in the regulation of the IL-2 and IL-2R genes, also remains activated. Therefore, whereas in mixed cultures, monocyte-dependent T cell activation and subsequent IL-2/IL-2R-mediated proliferation of T cells may be inhibited by anti-MHC class II Abs, our data clearly show that there also exists a direct way by which anti-MHC class II Abs can inhibit T cell proliferation which does not involve the T cell activation signal pathway. Indeed, it would be difficult to imagine how the inhibition of the signal pathway that results in IL-2 and IL-2R gene expression could result in such rapid arrest of proliferation, especially considering the fact both genes are already expressed at the time Tpi T cells are exposed to the Abs.

The nature of the inhibitory pathway is still poorly understood. In other systems, Ab binding to MHC class II molecules has been shown to cause changes in [Ca2+] and also altered serine/threonine-specific PKC (10) and protein tyrosine kinase activity (6, 9). In agreement with these previous studies, we could show that the PKC inhibitor H7 and the calcium chelator EGTA prevented anti-MHC class II-mediated aggregation of Tpi T cells. These inhibitors, however, did not prevent the arrest of DNA synthesis. The tyrosine kinase inhibitors herbimycin A and genistein, on the other hand, partly abrogated inhibition, whereas the phosphatase
inhibitor sodium orthovanadate enhanced inhibition. These preliminary data suggest that at least two separate signaling pathways are triggered by MHC class II engagement: a PKC-dependent pathway that results in arrest of DNA synthesis. In this regard, our observations resemble those for the anti-IgM-mediated inhibition of a human B cell lymphoma which was also shown to be independent of PKC activation and involved tyrosine phosphorylation (29).

In this case, herbimycin A and genistein also reversed anti-IgM-induced inhibition of growth. The signaling resulting from MHC class II engagement is complex and there is evidence that the participation of other surface molecules may determine whether MHC class II engagement will enhance or inhibit proliferation. This way, it was shown by one group that the anti-MHC class II mAbs Q5/6 and Q5/13 could enhance T cell proliferation induced via the CD2 molecule (30), whereas in another report, T cells treated with anti-CD3 Abs were inhibited by the same anti-MHC class II Abs (12, 19). A recent report (31), however, also showed that anti-MHC class II Abs could synergize with submictogenic amounts of anti-CD3 mAb in causing proliferation and lymphokine secretion. Such opposite activities have also been demonstrated for Abs that bind to other lymphocyte surface ligands (32–34).

The fact that cells inhibited by anti-MHC class II Abs are arrested in all phases of the cell cycle is puzzling, but not without precedent since non-phase–specific cell cycle arrest is also induced by anti-MHC class II Abs in B cells (11). The mechanism by which this is achieved is up till now entirely unknown, but MHC class II–mediated inhibition of Tpi T and also B cell proliferation (11) clearly differs from the irreversible arrest in G1-S induced by anti-CD3 and anti-Thy-1 Abs (33).

The fact that T cells that were cured of the parasite were also inhibited would indicate that anti-MHC class II–mediated inhibition of proliferation is not restricted to T cell clones that harbor *T. parva*. Although we do not want to generalize, the notion that Abs directed against MHC class II molecules can directly inhibit the proliferation of activated T cells is of considerable relevance, in particular, since such Abs can be detected in patients with autoimmune disease such as SLE and rheumatoid arthritis (35, 36). In this regard, we have found in preliminary experiments that sera from several patients with SLE potently inhibited the proliferation of Tpi T cells (Eichhorn, M., and D. A. E. Dobbelare, unpublished observations). In addition, evidence exists that Abs reactive with MHC class II antigens are generated in patients infected with HIV, apparently owing to amino acid sequence similarities between MHC class II and certain HIV-derived peptides (37–39). This has led to the proposal that autoimmune responses against HLA MHC class II molecules could lead to the progressive deterioration of the immune system. In the latter context, it has been shown that certain HIV envelope proteins are capable of activating T cells (40). An intriguing corollary of the fact that these HIV peptides can induce T cell activation, and thus MHC class II expression, is that this could predispose such cells to the inhibitory effects of the anti-MHC class II Abs.

Further work will be required, however, to determine whether inhibitory mechanisms similar to the one described in this paper are also involved in the pathogenesis of autoimmune diseases and AIDS.

We thank Isabel Roditi and Rolf Zinkernagel for critical review of the manuscript, and Richard Williams and Peter Herrlich for continued support. Ray Reeves and Angelika Ehrfeld are thanked for the bovine IL-2R/Tac and actin cDNA probes.

This work was supported by grants from the Deutsche Gesellschaft für Technische Zusammenarbeit (87.7860.7.100) and the Swiss National Science Foundation (Nr. 31.32.617.91).

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Received for publication 17 February 1993 and in revised form 5 May 1993.

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