Immunology 1990 **70** 440–445

Differential requirement for protein synthesis in cytolysis mediated by class I and class II MHC-restricted cytotoxic T cells

J. P. TITE *Department of Molecular Biology, Wellcome Biotech, Beckenham, Kent*

Accepted for publication 9 April 1990

SUMMARY

Antigen-specific cytolysis by major histocompatibility complex (MHC) class II-restricted cloned T-cell lines was found to be dependent on protein synthesis. Cytolysis by both polyclonal short-term and long-term class I-restricted cytotoxic T lymphocytes (CTL) was almost completely insensitive to inhibitors of protein synthesis. Kinetic studies with class II-restricted CTL indicated that approximately 2–3 hr after initiation of activation, resistance to inhibitors of protein synthesis was acquired. This strongly suggests that either a cytotoxic effector molecule or an intermediary important in the delivery of such a molecule is synthesized rapidly after activation in class II-restricted CTL, whilst class I-restricted CTL have no such requirement.

INTRODUCTION

Cytotoxic T cells are considered to be an important arm of the immune response in the battle against infection. Cytotoxic T cells specific for virally infected cells are generated during most viral infections, and analysis of this phenomenon led to one of the seminal discoveries in modern immunology, namely that of the major histocompatibility complex (MHC) restriction of T-cell responsiveness (Zinkernagel & Doherty, 1974). However, although the study of cytotoxic T cells has been extremely revealing in terms of T-cell recognition of antigen, only recently has limited progress been made in the elucidation of the actual molecular mechanism by which cytotoxic T lymphocytes (CTL) kill their targets. Early studies indicated that target cells were programmed to lyse within minutes of contact with the cytolytic effector (Martz & Benacerraf, 1973). Inhibitors of protein and RNA synthesis had no effect on the delivery of the so-called lethal hit (Thorn & Henney, 1976), suggesting that the cytolytic mechanism was already present in the effector population at the time of contact with the target cells. Another characteristic of the killing event was that third-party or innocent bystander target cells were spared from cytolysis (Martz, 1977), suggesting that the lethal hit was an extremely local event and not due to secretion of a widely diffusible factor.

For many years it was thought that T-cell-mediated cytotoxicity was the exclusive domain of class I MHC-restricted $CD8^+$ or Lyt-2^+ T lymphocytes. However, it is now apparent that class II MHC-restricted CTL are generated during immune responses against alloantigens (Swain *et al.*, 1981), soluble protein antigens (Tite & Janeway, 1984) and viral antigens (Lukacher *et al.*, 1985). Experiments with ovalbumin-specific $CD4^+$ class II MHC-restricted CTL indicated that when stimulated to kill antigen-specific targets, innocent bystanders were also lysed (Tite & Janeway, 1984; Tite, Powell & Ruddle, 1985). This suggested that a diffusible mediator was responsible for cytolysis. Analysis of supernatants from a panel of cytolytic and non-cytolytic T-cell clones revealed that all cytolytic T-cell clones secreted lymphotoxin and interferon-gamma (IFN-γ), whereas the non-cytotoxic T-cell clones did not (Tite *et al.*, 1985). Subsequently, it has been postulated that $CD4^+$ T-cell clones can be subdivided into two groups, Th1 cells, which secrete interleukin-2 (IL-2) and IFN-γ upon activation, and Th2 cells, which secrete IL-4 upon activation. The cytolytic $CD4^+$ T cells therefore fall within the Th1 subclass, and have also been termed inflammatory cells due to their ability to transfer delayed-type hypersensitivity (DTH). This report investigates the metabolic requirements of killing by both class I- and class II-restricted CTL. The results indicate that, in contrast to class I MHC-restricted CTL, class II MHC-restricted CTL require both mRNA and protein synthesis before becoming cytolytically active. Thus about 2–3 hr are required before the cytolytic process becomes insensitive to inhibitors of these events. Once activated, however, the cells are non-specifically cytotoxic and are insensitive to these inhibitors. The phase of non-specific cytotoxicity wanes by 24 hr, but the capacity to lyse in antigen-specific fashion is still present, indicating that reactivation can occur. It appears that synthesis of either a cytotoxic mediator or a protein required for its release or secretion is necessary for $CD4^+$ T cells to become cytolytically active.

Correspondence: Dr J. P. Tite, Dept. of Molecular Biology, Wellcome Biotech, Langley Court, Beckenham, Kent BR3 3BS, U.K.

MATERIALS AND METHODS

Animals

B10.S and BALB/c female mice were used as a source of splenic feeder cells for maintenance of T-cell lines and clones. Both strains were bred in the animal facility at Wellcome Biotech.

Antigens, viruses and peptides

Ovalbumin was purchased from Sigma Chemicals (Poole, Dorset). Influenza viruses used for the weekly restimulation of T-cell lines were grown in embryonated chicken eggs and either allantoic fluid or virus purified by ultracentrifugation (Skehel & Schild, 1971) used as an antigen source. Peptides were synthesized according to the method of Houghten (1985).

Other reagents

Phorbol myristate acetate (PMA), ionophore A23187, cycloheximide and actinomycin D were obtained from Sigma Chemicals. Recombinant murine IL-2 was purchased from Genzyme (Boston, MA).

Generation and maintenance of T-cell lines and clones

The generation of the cloned ovalbumin-specific CD4+, class II MHC-restricted T-cell line 5-8 has been described elsewhere (Tite & Janeway, 1984). B10.S T-cell clones responding to the major Th epitope (amino acids 270–285) in influenza A nucleoprotein (Gao, Liew & Tite, 1989) were generated by limiting dilution of a virus-specific T-cell line derived from nucleoprotein (NP)-immune mice and maintained with weekly feeding with irradiated syngeneic spleen cells and 1 μg/ml A/PR8 influenza virus. A long-term polyclonal CTL line with specificity for the major nucleoprotein class I CTL epitope (Taylor *et al.*, 1987) in BALB/c mice (amino acids 147–161) was generated from the spleen cells of influenza-infected mice by weekly restimulation with virus-infected, irradiated syngeneic spleen cells. All T-cell lines and clones were maintained in medium containing 10% of a supernatant from concanavalin A (Con A)-stimulated rat spleen cells. Short-term polyclonal influenza-specific CTL were generated as described elsewhere (Townsend & Skehel, 1984).

^{51}Cr-release assays

Antigen-specific ^{51}Cr-release assays with 5-8 T cells were performed using ^{51}Cr-labelled A20.2J target cells prepulsed overnight with 5 mg/ml ovalbumin. Unpulsed ^{51}Cr-labelled A20.2J cells were used to assess non-specific cytotoxicity. For B10.S class II-restricted T-cell clones and BALB/c class I-restricted cell lines specific for nucleoprotein peptides, target cells [LS102.9 (Tite *et al.*, 1985) or P815, respectively] were pulsed with peptide at 100 μg/ml at the same time as ^{51}Cr labelling. All target cells were as described in Tite *et al.* (1985).

RESULTS

Class II MHC-restricted CTL can be activated to kill by PMA plus ionophore

The 5-8 cloned T-cell line was activated for 4 hr with 30 nM PMA and 1 μM A23187, after which time the cells were washed and tested for cytotoxicity against a range of ^{51}Cr-labelled targets. High levels of cytotoxicity were observed against both the A20.2J and P815 tumour cell targets, but only low levels against three other target cells tested, namely 70/Z.3.12, BW5147 and EL4 (Table 1). This differential susceptibility is similar to that seen when lectin was used to activate cytotoxicity in class II MHC-restricted CTL (Tite *et al.*, 1985). The non-specific effector phase lasted less than 24 hr, at which time no killing was observed on A20.2J cells (J. P. Tite, unpublished observations).

Table 1. Analysis of tumour cell sensitivity to cytolysis by activated CD4 CTL

	% specific ^{51}Cr-release after incubation of T cells with:	
Target cells	Nil	PMA + A23187
A20.2J	1·6	65·4
P815	11·3	54·2
70Z/3·12	10·9	24·5
BW5147	5·7	20·1
EL4	1·2	4·7

5-8 T cells were incubated in the presence or absence of 30 nM PMA and 1 μM A23187 for 4 hr before washing and reculturing with the indicated radiolabelled tumour cell targets. $1{\cdot}2 \times 10^4$ T cells were incubated with 10^4 target cells for 6 hr, after which time supernatants were harvested to assess ^{51}Cr release.

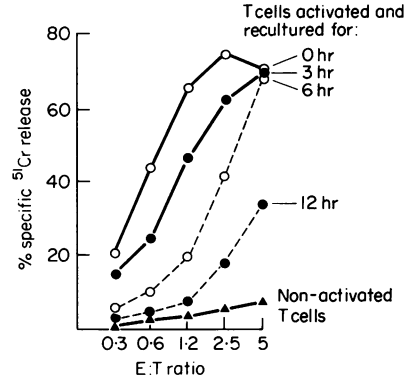

Figure 1. 5-8 cloned T cells were incubated for 4 hr with 30 nM PMA and 1 μM A23187 before washing and reculturing for the indicated lengths of time. The activated cells were then titrated against ^{51}Cr-labelled A20.2J target cells for 6 hr.

Figure 1 shows the decay of the non-specific cytotoxic effect; by 3 hr cytotoxicity had decayed by 50% and by 12 hr by 90%. The cells had not lost the ability to lyse targets specifically, however, since antigen-pulsed targets were readily lysed at this time (data not shown).

Requirement for protein synthesis in the generation of specific and non-specific cytotoxicity

The ability to generate non-specific cytotoxicity in class II MHC-restricted CTL with a combination of PMA plus A23187 allows the cytotoxic event to be analysed as two distinct stages, i.e. activation and the subsequent lytic event. Figure 2 indicates that the inhibitor of protein synthesis, cycloheximide, efficiently inhibited cytotoxicity by the 5-8 cloned T-cell line if present during both activation and lytic phases of the process; the presence of cycloheximide in either stage alone had only marginal effects on ^{51}Cr release. Other experiments showed that specific killing of ovalbumin-pulsed targets was also inhibited by

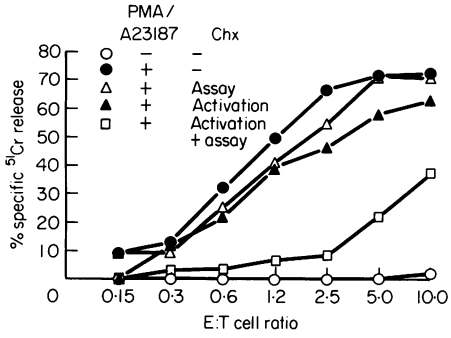

Figure 2. 5-8 cloned T cells were activated for 4 hr with 30 nM PMA and 1 μM A23187. Cycloheximide (Chx) was added at a final concentration of 5 μg/ml in either the activation culture or ^{51}Cr-release assay culture or both as indicated.

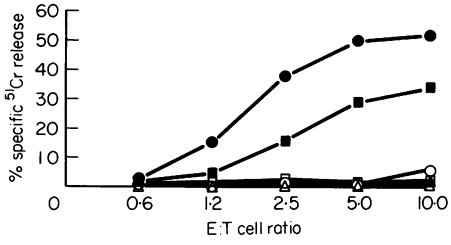

Figure 3. 5-8 cloned T cells were activated for lysis by co-culture with 10^4 A20.2J cells pulsed with ovalbumin (OVA) (5 mg/ml for 18 hr) (solid symbols) or 10^4 A20.2J cells (open symbols). Activation cultures were incubated for 4 hr, after which time ^{51}Cr-release against ^{51}Cr-labelled A20.2J cells was measured. Cycloheximide at 5 μg/ml was added to either both the activation and assay stage or assay stage alone. ^{51}Cr release was measured after a further 6 hr incubation. (●) Preculture with ovalbumin/A20.2J, no cycloheximide; (■) preculture with ovalbumin/A20.2J, cycloheximide in assay only; (▲) preculture with ovalbumin/A20-2J, cycloheximide in activation plus assay. Preculture with unpulsed A20-2J under same conditions (○, □, △).

cycloheximide (data not shown; Fig. 4). In this case, presence in the ^{51}Cr-release assay was sufficient to cause inhibition, reflecting the fact that activation and lysis are occurring simultaneously in the ^{51}Cr-release culture in this system. If the specific activation of killing is performed by preincubating the effector cells with non-radiolabelled ovalbumin-pulsed A20.2J cells for 4 hr before the addition of radiolabelled non-pulsed target cells, then cycloheximide is required in both the assay and preactivation to obtain complete inhibition (Fig. 3). Cycloheximide present during the ^{51}Cr-release assay led to only marginal inhibition.

Lysis by class I MHC-restricted CTL is not inhibitable with cycloheximide

The observation that protein synthesis is required by class II MHC-restricted CTL during lysis is in contrast with early studies with class I MHC-restricted CTL. Figure 4 indicates that specific killing by either a class I-restricted influenza-specific CTL line or by polyclonal influenza-specific CTL was only marginally (< 50%) inhibited by cycloheximide, whereas killing by a cloned influenza-specific class II-restricted T-cell line and

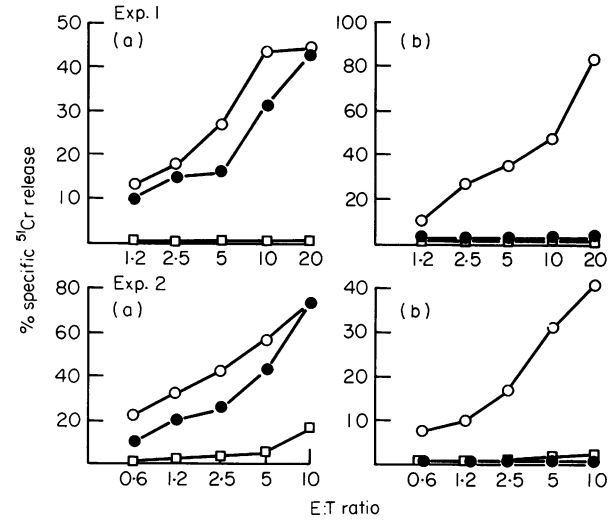

Figure 4. Cytolytic effectors were titrated against target cells pulsed with appropriate peptides in the presence or absence of 5 μg/ml cycloheximide. Exp. 1. (a) A long-term BALB/c influenza-specific line reactive to amino acids 147–161 of influenza NP was titrated against (○) amino acid 147–161 (100 μg/ml)-pulsed P815 cells; (●) amino acid 147–161-pulsed P815 + 5 μg/ml cycloheximide; (□) unpulsed P815. (b) A long-term B10.S class II-restricted T-cell line specific for influenza NP and recognizing amino acids 260–283 of influenza NP was titrated against (○) amino acid 260–183-pulsed LS102.9 cells; (●) amino acid 260–283-pulsed LS102.9 + 5 μg/ml cycloneximide; (□) unpulsed LS102.9. Exp. 2. Same as Exp. 1 but effector cells in (a) were polyclonal effector T cells taken 5 days after secondary *in vitro* activation of influenza-immune BALB/c spleen cells, and in (b) effectors were cloned class II-restricted B10.S T cells responding to amino acids 260–283 of NP.

an uncloned cell line of similar specificity and characteristics was completely inhibited in the same experiments. In both cases, cytotoxicity was tested on target cells pulsed with short synthetic peptides defining either class I- or class II-restricted epitopes of the influenza nucleoprotein molecule. This rules out differential effects of cycloheximide on processing of antigen for class I or class II MHC-restricted recognition.

Inhibition of cytolysis by class II MHC-restricted CTL by actinomycin D

The inhibitor of RNA synthesis, actinomycin D, was used to test the requirement for the synthesis of messenger RNA in the killing by class II MHC-restricted CTL. Figure 5a indicates that killing of ovalbumin-pulsed target cells by 5-8 T cells was inhibited with equal efficiency by actinomycin D and cycloheximide when these drugs were added to cultures at the initiation of the ^{51}Cr-release assay. An approximately 30-fold reduction in cytolysis was observed with both drugs. Incubation of effector cells with actinomycin D for longer periods before addition of targets led to even more profound inhibition of cytolysis (data not shown). These data suggest that mRNA synthesis is required for the generation of the protein moiety(s) involved in killing and that control is at the transcriptional and not at the translational level. Actinomycin D, however, had no effect on the killing by cells preactivated with PMA plus A23187 (Fig. 5b). Inclusion of the drug in the activation cultures did, however, prevent cloned T cells from becoming non-specifically cytotoxic. Thus for both specific and non-specific cytotoxicity there is a requirement for RNA synthesis.

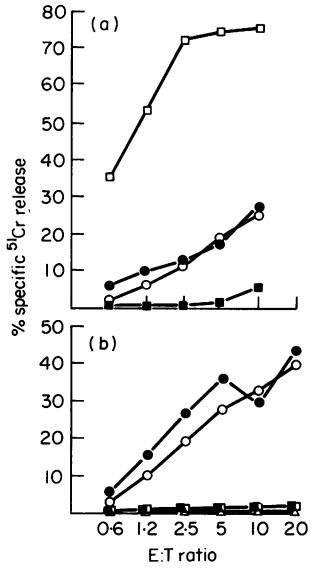

Figure 5. (a) 5-8 cloned T cells were titrated against ^{51}Cr-labelled ovalbumin/A20.2J cells in the presence of either medium (□), cycloheximide (5 μg/ml) (●) or actinomycin D (5 μg/ml) (○). Unpulsed A20.2J were also included (■). (b) 5-8 T cells were activated for 4 hr with 30 nM PMA and 1 μM A23187. Actinomycin D was added to either the subsequent ^{51}Cr-release assay stage (●) or to the activation stage alone (□) or to both (■). Ovalbumin/A20.2J cells in the absence of actinomycin (○); A20.2J cells in the absence of actinomycin (△).

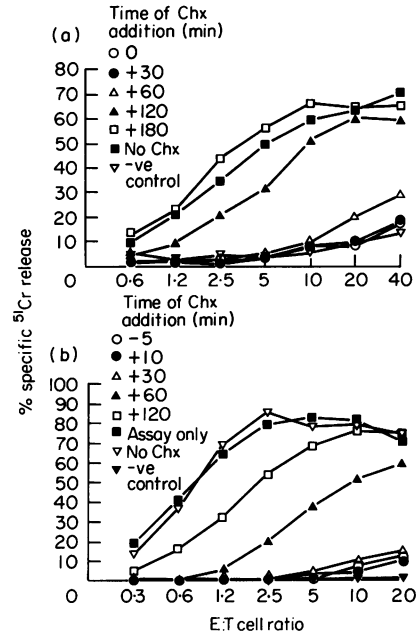

Figure 6. (a) 5-8 cloned T cells were titrated against ^{51}Cr-labelled ovalbumin/A20.2J cells for 6 hr. Cycloheximide (Chx) at 5 μg/ml was added at the indicated times after the initiation of culture. (b) 5-8 cloned T cells were activated for 3 hr with PMA/A23187. Cycloheximide (5 μg/ml) was added at the indicated times. After activation, cycloheximide was also added to the assay cultures at 5 μg/ml as indicated. Control cultures containing no cycloheximide or cycloheximide in the assay only were also set up.

Kinetics of activation for cytotoxicity

In order to determine the precise timing of the requirement for protein synthesis during the cytolytic process, experiments were performed using cycloheximide to inhibit protein synthesis at varying times after the activation of cells. Figure 6a shows an experiment in which antigen-specific cytolysis of ovalbumin-pulsed A20.2J cells by the 5-8 T-cell line was studied. It is clear that addition of cycloheximide after 120 min does not inhibit cytolysis significantly, whereas addition before this time leads to profound inhibition. A similar result was obtained when activation was achieved with PMA/A23187 (Fig. 6b). Addition of cycloheximide 60 min after initiation of cultures gave an approximately 90% reduction in cytolysis, addition at 120 min gave a 50% reduction and addition during the assay only, i.e. at 180 min, gave no inhibition. A similar pattern was observed when the kinetics were analysed using EGTA to delete Ca^{2+} from the medium (data not shown). EGTA was effective at inhibiting antigen-specific cytolysis at times up to 120 min, after which addition of EGTA had no effect. Addition of EGTA had no effect on killing by T cells preactivated with PMA/A23187 (J. P. Tite, unpublished observations).

Inhibition of killing by cycloheximide is not due to inhibition of IL-2 synthesis

Non-specific cytolysis by class II MHC-restricted CTL can be induced by incubation with recombinant IL-2 (rIL-2) (J. P. Tite, unpublished observations). A possible explanation for the inhibition of cytolysis by cycloheximide is that production of IL-2 is being inhibited and this prevents the induction of a non-specific effector phase. This appears not to be the case, as the induction of non-specific killing by rIL-2 was inhibited by

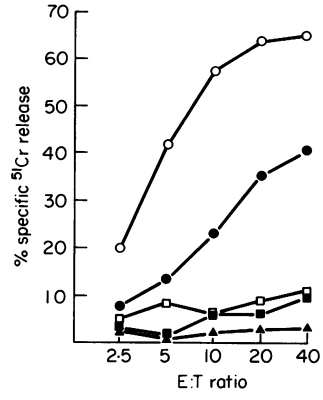

Figure 7. 5-8 T cells were co-cultured with either ovalbumin/A20.2J ^{51}Cr targets (○) or A20.2J ^{51}Cr targets (■) without further addition. Some cultures with A20.2J targets received 20 U/ml recombinant murine IL-2 either with 5 μg/ml cycloheximide (▲) or with medium (●). Cultures with ovalbumin/A20.2J targets plus cycloheximide were also included (□).

cycloheximide (Fig. 7) and the addition of rIL-2 did not reverse the inhibition of non-specific killing by cycloheximide (data not shown). Synthesis of IL-2 does not seem to be the limiting event in cytolysis.

DISCUSSION

The initial discovery of cytolytic T cells led to intense research on the mechanism by which cytolysis is effected. Much data on

the kinetics of lysis, requirement for protein and mRNA synthesis and cationic metal ions were accumulated, but details of the molecular mechanism remained elusive (Martz, 1977). More recently there have been several proposals for candidate molecules involved in cytolysis. Podack & Konigsberg (1984) have described granules present in several types of cytolytic effector which when isolated are capable of lysing targets. One component of these granules is a pre-forming protein called perforin which shows structural homology with the C9 component of complement. Recently, however, evidence has accrued which indicates that granules and perforins are not found in short-term effector CTL populations, but develop only after long-term *in vitro* cultivation (Dennert, Anderson & Prochazka, 1987). Another potential candidate for participation in the cytotoxic machinery of CTL is a family of serine esterases (Pasternak & Eisen, 1985), subsequently shown to be present in CTL granules (Masson & Tschopp, 1987). The stimulation of cytotoxic T cells by target cells or/and T-cell receptor antibodies leads to exocytosis of these esterases from the CTL. However, there is compelling evidence to suggest that these enzymes are not absolutely essential for killing, namely the ability to divorce exocytosis of enzyme and the cytotoxic act in some circumstances (Ostergaard *et al.*, 1987) and the ability to generate CTL clones devoid of esterase activity (Brunet *et al.*, 1987). Finally, there has been a report of a tumour necrosis factor (TNF)-like molecule which may be involved in target cell lysis by class I MHC-restricted CTL (Liu *et al.*, 1987). In all probability, there are several mechanisms by which CTL effect their cytotoxicity and the full picture is only slowly being composed. We have concentrated on the phenomenon of killing by $CD4^+$ T cells restricted to class II MHC gene products. Early studies indicated that such cells were capable of both direct lysis of antigen-pulsed, class II MHC-positive tumour cells and also bystander lysis of cells present in the culture of effector and specific targets. Analysis of the lymphokines secreted by these $L3T4^+$ CTL indicated that they produced IFN-γ and lymphotoxin and it is now clear that they are representatives of the Th1 (Mosmann *et al.*, 1986) or inflammatory helper T-cell subset (Bottomly, 1988). Since our initial observations, other groups have also tested $L3T4^+$ T-cell clones and found them to have both direct and bystander cytolytic activity (Ju *et al.*, 1989). One possible explanation for this cytolytic activity is that the cells produce, on specific stimulation, a cytolytic mediator which non-specifically kills cells, those being most proximal being first affected. This report studied the effect of inhibitors of protein synthesis and RNA synthesis on the activation and effector phase of cytotoxicity. It is concluded from these experiments that a protein synthetic event is required for class II MHC-restricted T cells to become cytolytically active. This is in strict contrast to class I MHC-restricted CTL, which are unaffected by cycloheximide treatment. It would appear that class I MHC-restricted CTL, whether from short- or long-term cultures, have effector molecules and the mechanism to release them already synthesized. Class II-restricted CTL, by contrast, need either to synthesize effector molecules or possibly other molecules needed for release and/or delivery of such effector molecules. It is unlikely that cycloheximide differentially affects events in the target cell after receiving the cytolytic signal, as killing by preactivated class II-restricted CTL is not affected by cycloheximide. The synthesis of the essential protein moiety is also dependent on the synthesis of mRNA since cytolysis is also inhibited by actinomycin D. IL-2 does not appear to be the critical cytokine as exogenous rIL-2 does not reverse the inhibition by cycloheximide and rIL-2-induced killing is strongly inhibited by cycloheximide. Other candidates for the newly synthesized lytic machinery are the cytokines TNF-α and TNF-β. It was not possible to inhibit cytolysis with an antiserum specific for murine TNF-α, which is capable of inhibiting the cytolytic effects of recombinant TNF-α. (J. P. Tite, unpublished observations). However, in an accompanying paper (Tite, 1990), it is shown that variants of cell lines selected for resistance to killing by TNF-α were also rendered insensitive to killing by class II MHC-restricted CTL, suggesting that there is a common link between the two killing mechanisms. The TNF-resistant cells were indistinguishable from the parental cell line in terms of sensitivity to class I MHC-restricted effector cells. These data do not rule out the possibility that $CD4^+$ CTL possess both TNF-dependent and TNF-independent mechanisms of cytolysis, as suggested by the data of Ju *et al.* (1989). Further experiments are in progress to investigate this possibility.

In conclusion, it is shown that class II MHC-restricted, $CD4^+$ CTL have requirements distinct from $CD8^+$ CTL; kinetic studies indicate that for $CD4^+$ CTL 2–3 hr after initial T-cell stimulation are required before cytolytically active cells can be detected. This is sufficient time for *de novo* synthesis and translation of mRNA for a putative mediator. Thus far this mediator has not been identified. If the mediator could be identified, it may be an important drug target as $CD4^+$ CTL similar to those described here are thought to be important in several autoimmune disease states (Sun & Wekerle, 1986).

REFERENCES

Bottomly K. (1988) A functional dichotomy in $CD4^+$ T lymphocytes. *Immunol. Today*, **9**, 268.

Brunet J.F., Denizok F., Suzan M., Haas W., Mencia-Huerta J.-M., Berke G., Luccni M.F. & Golstein P. (1987) CTL-A and CTLA-3 serine esterase transcripts are detected mostly in cytotoxic T-cells, but not only and not always. *J. Immunol.* **138**, 4102.

Dennert G., Anderson C.G. & Prochazka G. (1987) High activity of serine esterase and cytolytic perforin in cloned cell lines is not demonstrable in *in vivo* induced cytotoxic effector cells. *Proc. natl. Acad. Sci. U.S.A.* **84**, 5004.

Gao X.-M., Liew F.Y. & Tite J.P. (1989) Identification and characterisation of T-helper epitopes in the nucleoprotein of influenza A virus. *J. Immunol.* **143**, 3007.

Houghten R.A. (1985) General method for the rapid solid phase synthesis of a large number of peptides: specificity of antigen-antibody interaction at the level of the individual amino acid. *Proc. natl. Acad. Sci. U.S.A.* **82**, 183.

Ju S.-T., Strack P., Stromquist D. & Dekruyff R.M. (1989) Cytolytic activity of Ia-restricted T-cell clones and hybridomas: evidence for a cytolytic mechanism independent of interferon-gamma, lymphotoxin and tumor necrosis factor-α. *Cell Immunol.* **117**, 399.

Lui C.-C., Steffan M., King K. & Young J.D.E. (1987) Identification, isolation and characterisation in murine cytolytic lymphocytes. *Cell*, **51**, 393.

Lukacher E., Morrison L.A., Braciale V.L., Malissen B. & Braciale T. (1985) Expression of specific cytolytic activity by H2-I region-restricted, influenza virus-specific T lymphocytes clones. *J. exp. Med.* **162**, 171.

Martz E. (1977) Mechanisms of specific tumor cell lysis by alloimmune T lymphocyte, resolution and characterisation of discrete steps in the cellular interaction. *Contempt. Top. Immunobiol.* **7**, 301.

MARTZ E. & BENACERRAF B. (1973) An effector cell independent step in target cell lysis by sensitixed mouse lymphocytes. *J. Immunol.* **111,** 1538.

MASSON D. & TSCHOPP J. (1987) A family of serine esterases in lytic granules of cytolytic T lymphocytes. *Cell,* **49,** 679.

MOSMANN T., CHERWINSKE H., BOND M.W., GIEDLIN M.A. & COFFMAN R.L. (1986) Two types of murine helper T-cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *J. Immunol.* **136,** 2348.

OSTERGAARD H.L., KANE K.P., MESCHER M.F. & CLARK W.R. (1987) Cytotoxic T lymphocyte mediated lysis without release of serine esterase. *Nature (Lond.),* **330,** 71.

PASTERNAK M.S. & EISEN H.N. (1985) A novel serine esterase expressed by cytotoxic lymphocytes. *Nature (Lond.),* **314,** 743.

PODACK E.R. & KONIGSBERG P.J. (1984) Cytolytic T-cell granules: isolation, structural, biochemical and functional characterisation. *J. exp. Med.* **160,** 695.

SKEHEL J.J. & SCHILD G. (1971) The polypeptide composition of influenza A viruses. *Virology,* **44,** 396.

SUN D. & WEKERLE H. (1986) Ia-restructed encephalitogenic T lymphocytes mediating EAE lyse autoantigen-presenting astrocytes. *Nature (Lond.),* **320,** 70.

SWAIN S.L. *et al.* (1981) The Lyt phenotype of a long-term allospecific T-cell line. Both helper and killer activities are mediated by Lyt-1 cells. *Eur. J. Immunol.* **11,** 175.

TAYLOR P.M., DAVEY J., HOWLAND K., ROTHBARD J.B. & ASKONAS B.A. (1987) Class I MHC molecules rather than any other mouse genes dictate influenza epitope recognition by cytotoxic T-cells. *Immunogenetics,* **26,** 207.

THORN R.M. & HENNEY C.S. (1976) Studies on the mechanism of lymphocyte mediated cytolysis. VI. A reappraisal of the requirement for protein synthesis during T-cell-mediated lysis. *J. Immunol.* **116,** 146.

TITE J.P. (1990) Evidence of a role for TNF-α in cytolysis by $CD4^+$, class II MHC-restricted cytotoxic T cells. *Immunology,* **71,** (in press).

TITE J.P. & JANEWAY C.A. (1984) Cloned helper T-cells can kill B-lymphoma cells in the presence of specific antigen: Ia-restriction and cognate vs non-cognate interactions in cytolysis. *Eur. J. Immunol.* **14,** 878.

TITE J.P., POWELL M.B. & RUDDLE N.H. (1985) Protein–antigen specific Ia-restricted cytolytic T-cells: analysis of frequency, target cell susceptibility and mechanism of cytotoxicity. *J. Immunol.* **135,** 25.

TOWNSEND A.R.M. & SKEHEL J.J. (1984) The influenza A virus nucleoprotein gene controls the induction of both subtype specific and cross-reactive cytotoxic T-cells. *J. exp. Med.* **160,** 552.

ZINKERNAGEL R.M. & DOHERTY P.C. (1974) Restriction of *in vitro* T-cell-mediated cytotoxicity in lymphocytic choriomeningitis within a syngeneic or semi-syngeneic system. *Nature (Lond.),* **248,** 701.