Immunology 1988 **64** 129–134

Appearance of granule-associated molecules during activation of cytolytic T-lymphocyte precursors by defined stimuli

J. A. GARCIA-SANZ,* F. VELOTTI,* H. R. MACDONALD,† D. MASSON,‡ J. TSCHOPP‡ & M. NABHOLZ*

**Genetics Unit, Swiss Institute for Experimental Cancer Research, †Ludwig Institute for Cancer Research, Lausanne Branch and ‡Institute of Biochemistry, University of Lausanne, Epalinges, Switzerland*

Accepted for publication 7 December 1987

SUMMARY

Lysis of target cells by cytolytic T lymphocytes (CTL) is associated with the exocytosis of cytoplasmic granules. Purified granules from CTL cell lines contain a pore-forming protein (perforin), tree serine esterases, granzyme A (60,000 MW), granzyme B (29,000 MW), and granzyme C (27,000 MW). We have compared the kinetics of appearance of cytolytic activity with that of perforin and granzyme A activity during activation of lymphocytes from normal animals with leukoagglutinin (LA) and recombinant interleukin-2 (rIL-2). Unstimulated lymph node cells do not express any of these activities, which appear between Day 3 and Day 4 of stimulation and increase rapidly to reach a pronounced peak on Day 6. On Day 7 all the activities are considerably lower, even though the cells still proliferate exponentially. There is a good correlation between the kinetics of appearance of all of these activities. Using antisera against perforin and against granzyme C, one can detect positive cytoplasmic granules in a small fraction of cells on Day 3; by Day 5, 80–90% of the cells are stained. This proportion decreases again on Day 7.

INTRODUCTION

The generation of cytolytic T lymphocytes (CTL) from their cytolytically inactive precursors is a process that normally involves, at least *in vitro*, both activation and proliferation (reviewed by Nabholz & MacDonald, 1983). Most murine CTL precursors (pCTL) belong to the Lyt-2$^+$ L3T4$^-$ subset of mature T cells. They are small non-dividing inactive cells that acquire cytolytic activity only after stimulation with antigen or mitogenic lectins. Erard *et al.* (1985a, c) recently showed that single Lyt-2$^+$ cells proliferate and become cytolytic in response to lectin or to allogeneic stimulation in the presence of recombinant IL-2 (rIL-2). Their activation does not require any other cells or exogenously added factors, whereas L3T4$^+$ cells need additional activation signals to become responsive to IL-2 (Erard *et al.* 1985b).

Recent studies on the mechanism of cell-mediated cytotoxicity using CTL cell lines strongly suggest that the antigen-specific interaction of a CTL with its target cell stimulates exocytosis of cytoplasmic granules that contain the proteins responsible for the delivery of the lethal hit (reviewed by Henkart, 1985). Purified cytoplasmic granules from CTL can lyse different cells, including erythrocytes, without specificity (Podack & Konigsberg, 1984) and contain a molecule which polymerizes in the target cell membrane to form tubular lesions that resemble those produced by the complement component C9 (Podack & Konigsberg, 1984, Masson *et al.*, 1985). This protein (referred to as perforin, cytolysin, pore-forming protein or C9-related protein) has been identified as the factor in the granules responsible for erythrocyte lysis (haemolysis) (Masson & Tschopp, 1985; Podack, Young & Cohn, 1985).

Granules with the same density as those containing perforin also contain several proteins that have been identified as serine esterases on the basis of their reaction with the serine esterase-specific inhibitor diisopropyl-fluorophosphate (DFP) and/or their *N*-terminal amino acid sequences (Pasternack & Eissen, 1985; Masson *et al.*, 1986a; Gershenfeld & Weissman, 1986; Masson, Zamai & Tschopp, 1986b; Kramer *et al.*, 1986; Masson & Tschopp, 1987). Among these, there are: granzyme A (also called BLT-esterase, TSP-1, SE-1, and molecularly cloned as H-factor), which consists of two disulphide-linked 35,000 MW subunits and cleaves the esterase substrate *N*-α-benzyloxycarbonyl-L-lysine thiobenzyl ester (BLT) (Masson *et al.*, 1986b), and granzyme B, another DFP-reactive, 29,000 MW molecule (Masson *et al.*, 1986a). A further granule-associated protein clearly belongs to the same serine esterase family on the basis of its strong sequence homology to both granzyme A and granzyme B (Masson & Tschopp, 1987; Jenne *et al.*, 1988). Therefore

Abbreviations: BLT, *N*-α-benzyl oxycarbonyl-L- lysine thiobenzylester; Con A, concanavalin A; CTL, cytolytic T lymphocytes; DFP, diisopropyl fluorophosphate; LA, leukoagglutinin; mAb, monoclonal antibody; PHA, phytohaemagglutinin; rIL-2, recombinant interleukin-2; TLCK, Tosyl-L-lysine chloromethylketone.

Correspondence: Dr J. A. Garcia-Sanz, Genetics Unit, ISREC, 155, ch. des Boveresses, CH-1066 Epalinges s/Lausanne, Switzerland.

this protein has been called granzyme C, although it does not bind DFP.

The present study represents the first investigation of the kinetics of appearance of perforin, granzyme A and granzyme C during activation of pCTL in a defined culture system.

MATERIALS AND METHODS

Mice

Adult female C57B1/6 and DBA/2 mice were obtained from the animal colony of the Swiss Institute for Experimental Cancer Research, Epalinges.

Interleukin-2

Purified recombinant IL-2 (rIL-2), of human origin (Devos *et al.*, 1983), was provided by Biogen SA, Geneva. The specific activity of the material was 0·3 ng protein/unit of biological activity.

Antisera

Two different antisera were used for immunocytochemical staining: a rat anti-granzyme C (anti-27,000 MW) serum raised by immunization with cytoplasmic granules (Masson *et al.*, 1985) and a rabbit antiserum raised against purified perforin. When tested against nitrocellulose blots of cytoplasmic granule proteins separated by SDS–PAGE, the two antisera react strongly with granzyme C and perforin, respectively, but there is some cross-reactivity with other granule constituents. The antisera do not react with nitrocellulose blots of cytoplasmic granule proteins from EL-4 cells separated by SDS–PAGE. In permeabilized CTL or CTL lines they stain exclusively cytoplasmic granules. They do not react with resting lymphocytes, the T-lymphoma cell line EL-4 or the mastocytoma cell line P815 (data not shown).

Cell cultures

Nylon-wool-purified C57B1/6 responder cells were prepared from lymph node cell suspensions from which erythrocytes had been removed by hypo-osmotic shock. The cells were cultured in MLC medium (Erard *et al.*, 1985a), containing 0·5 μg/ml leukoagglutinin (LA) (Pharmacia, Uppsala, Sweden) and 200 U/ml rIL-2 in 2-ml cultures (24-well plates Falcon, Becton-Dickinson, CA), at 37° in a humidified atmosphere of 5% CO_2 in air. The cultures to be harvested at different time-points were inoculated with different cell numbers so that they contained similar cell concentrations at the time of harvest. Harvest: Day 3, inoculum: $2{\cdot}5 \times 10^5$/ml; Day 4, 1×10^5/ml; Day 5, 5×10^4/ml; Day 6, 2×10^4/ml; Day 7, 1×10^4/ml.

^{51}Cr-release assay

^{51}Cr-release assay was done, as previously described (Erard *et al.*, 1985a), in the presence of lectin (2 μg/well Con A) which serves to reveal cytolytic activity independently of antigen recognition (Bevan, Langman & Cohn, 1976).

Assays for haemolytic and granzyme A activities

The assay for haemolytic activity in whole cell lysates was performed as previously described (Garcia-Sanz *et al.*, 1987). The activity of granzyme A was measured, as previously described (Garcia-Sanz *et al.*, 1987), by its capacity to hydrolize the substrate BLT in the presence of 1 mM TLCK. The relative amounts of perforin and granzyme A were determined by serial dilution of the extracts.

[3H]DFP labelling

Fifty microlitres of whole-cell lysates ($0{\cdot}5 \times 10^6$ cells) were incubated with 3 μl (15 μCi) of [^{3}H] DFP (Amersham, Amersham, Bucks, U.K.) during 1 hr at 37° (Masson *et al.*, 1986a). The samples were then boiled with 10 mM dithiothreitol (DTT) in sample buffer and loaded onto a 10% SDS–PAGE gel according to Laemmli (1970). [^{3}H] DFP-labelled proteins were detected by exposing a preflashed Kodak X-OMAT-film (Kodak, Rochester, NY) to the dried gel.

Immunoperoxidase staining

Cells were cytocentrifuged at 65 *g* for 5 min onto slides. The cells were air dried, fixed with acetone and incubated for 10 min at room temperature in 7·5% H_2O_2 diluted in PBS to block endogenous peroxidase, followed by two washes in PBS containing 5×10^{-5} M thimerosal (PBS-T).

For the staining a three-stage biotin-avidin-peroxidase system was used (Guesdon, Ternynck & Avrameas, 1979). The preparations were incubated with: (i) 25 μl of either 1/1000 diluted anti-perforin or 1/300 diluted anti-granzyme C antiserum for 60 min; (ii) 25 μl of the corresponding biotinylated anti-Ig antiserum (Vector Inc., Burlingame, CA) for 15 min; (iii) 25 μl of avidin horseradish peroxidase conjugate (HRP-avidin), 90 μl/ml (Vector Inc.), for 15 min. All incubations were performed at room temperature in a moist chamber, followed by three washes in cold PBS-T. The HRP was revealed by a 5-min incubation in the dark with freshly prepared 3,3′diaminobenzidine (0·5 mg/ml) (Sigma, St Louis, MO) dissolved in 0·05 M Tris-HCl buffer, pH 7·6, containing 0·015% H_2O_2 and counterstained with 1% OsO_4.

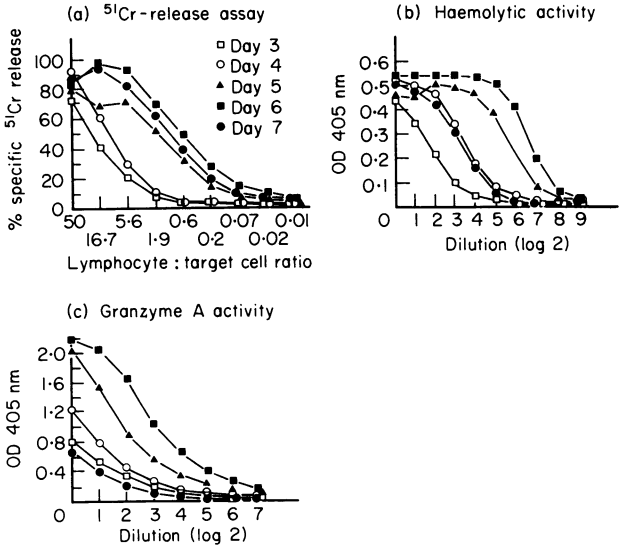

Figure 1. Cytolytic, perforin and granzyme A activity of mitogen-activated lymph node cells. Nylon-wool-purified lymph node cells were cultured at different cell concentrations in presence of leukoagglutinin (LA) 0·5 μg/ml and 200 U/ml rIL-2 to give exponentially growing cells, and harvested at different times. Aliquots of the cells were assayed for their cytolytic activity (a), as well as for the perforin (b) and granzyme A (c) contents. [The first dilution in (b) and (c) contains the extract of 10^6 cell.]

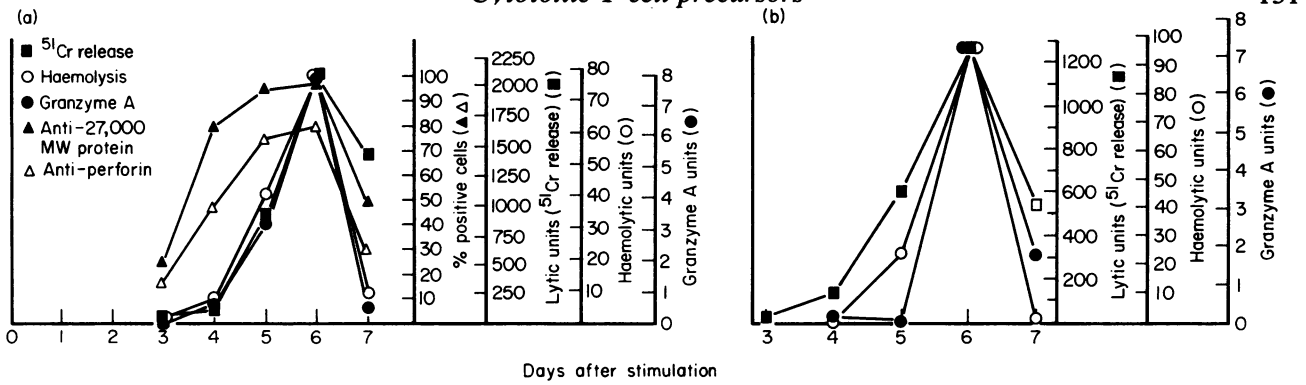

Figure 2. Kinetics of appearance of the different activities. The results from Fig. 1 (a), and an additional identical experiment (b), are plotted as units per million cells of the different activities. (a) also shows the percentage of cells stained with the anti-granzyme C (27,000 MW protein) and anti-perforin antisera in the same experiment. At least 200 cells were counted for each time point.

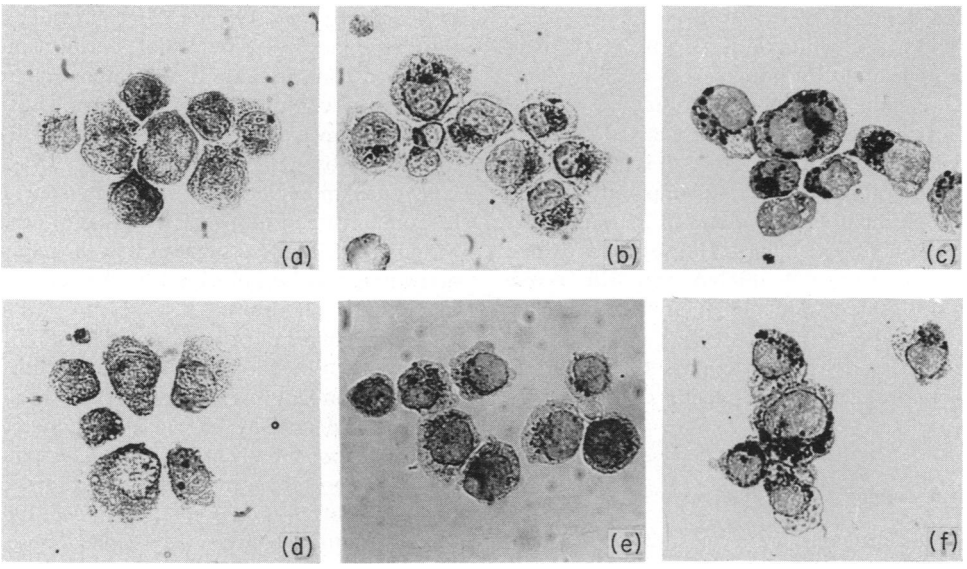

Figure 3. Detection of granule protein in leukoagglutinin-stimulated cells. Permeabilized mitogen-stimulated cells were stained (as described in the Materials and Methods) with the rat anti-granzyme C (anti-27,000 MW) antiserum (b and c), the rabbit anti-perforin antiserum (e and f), or the corresponding anti-Ig antisera alone (a and d). (b) and (e), cells after 3 days of stimulation; (a), (c), (d) (f), cells after 6 days of stimulation.

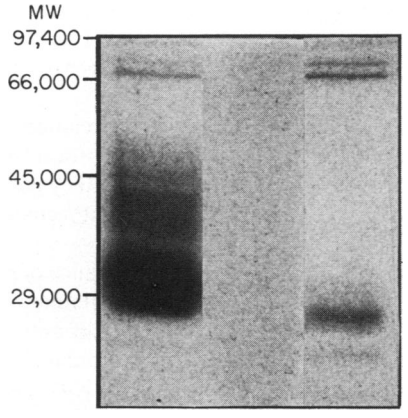

Figure 4. [^{3}H] DFP labelling of mitogen-activated lymph node cells. SDS–PAGE of whole cell lysates labelled with [^{3}H] DFP. Lysates of (a) CTL cell line B6.1 (from which granzyme A and granzyme B had been purified. (Masson *et al.*, 1986a), (b) unstimulated lymph node cells and (c) lectin-stimulated lymph node cells after 6 days of culture.

RESULTS

Appearance of cytolytic, haemolytic and granzyme A activities

Unstimulated lymph node cells have no detectable cytolytic, haemolytic or granzyme A activity (data not shown). They do not contain amounts of granzyme B that can be detected by labelling with [^{3}H] DFP (see below) and cannot be stained with the anti-27,000 MW (granzyme C) or the anti-perforin antiserum (data not shown).

Nylon-wool-purified lymph node cells were stimulated with LA and rIL-2, conditions that have been shown to stimulate specifically Lyt-2$^+$ cells (Erard *et al.*, 1985b). The percentage of L3T4$^+$ cells surviving on Day 6 (peak of the measured activities, see below) was less than 5% (data not shown) even though we used higher cell densities than in the previously described experiments. Under these conditions, the cells grow exponentially from Day 3, to at least Day 8 (data not shown).

After stimulation the different activities began to be detectable by Day 3 (Fig. 1) and increased rapidly to reach their peak

on Day 6 (Fig. 2). All the activities declined from Day 6 to 7 (Fig. 2), athough the cells were still proliferating exponentially.

In general, we found a good correlation between the day of first appearance and the time–course of the cytolytic, haemolytic and granzyme A activities (Fig. 2a), but in some experiments cytolytic activity was measurable before haemolytic or granzyme A activity (Fig. 2b).

The following lines of evidence indicate that the enzyme responsible for BLT-hydrolysis is indeed granzyme A.

(i) For CTL-cell lines the bulk of BLT hydrolysis is due to granzyme A [also called BLT-esterase (Pasternack & Eisen, 1985), TSP-1 (Kramer *et al.*, 1986), or SE-1 (Young *et al.*, 1986b)], which is present in granules with the same density as those containing perforin. There is no strong TLCK-resistant BLT-hydrolytic activity found in macrophages or B lymphocytes (Pasternack & Eisen, 1985; J. A. Garcia-Sanz and D. Masson, unpublished results)

(ii) In a T-cell hybrid, induction of BLT-hydrolytic activity correlates with the appearance of granzyme A mRNA (J. A. Garcia-Sanz and G. Plaetinck, unpublished results).

(iii) In normal T lymphocytes activated in a mixed leucocyte reaction, a BLT-hydrolytic activity appears that is contained in granules of the same density as those containing perforin, and during ion-exchange chromatography elutes in the same fractions as granzyme A from CTL-cell lines. The normal activated T cells also contain granzyme A mRNA and will secrete granzyme A upon antigenic stimulation (Garcia Sanz *et al.*, 1987; Velotti, MacDonald & Nabholz, 1987).

Together these data render it very unlikely that the BLT-hydrolytic activity of normal activated T cells is due to another enzyme.

Immunocytochemical staining

Using rat or rabbit antisera against granzyme C (27,000 protein) and against perforin we followed the appearance of cytoplasmic granules during activation of the cells with LA and IL-2 by staining the fixed, permeabilized cells. Unstimulated lymph node cells did not react with the antisera (not shown). With either reagent, positive cells were first detected on Day 3 of stimulation. As expected, the antibodies were localized over clearly defined areas in the cytoplasm of the positive cells. No staining of the cell surface was detectable. With both antisera the percentage of positive cells and the intensity of the staining increased from Day 3 (Figs 2a, and 3b,e) to Day 6 (Figs 3 c,f) and decreased again on Day 7 (Fig. 2a).

[^{3}H] DFP labelling

The appearance of the serine esterase granzyme B (29,000 MW DFP-reactive protein) was monitored by [^{3}H] DFP labelling of whole cell lysates followed by fractionation on SDS–PAGE. Figure 4 shows a comparison of the pattern of [^{3}H] DFP-labelled proteins in lysates from a CTL-line (B6.1) from which granzyme A (35,000 MW species) and granzyme B (29,000 MW species) were purified (Masson *et al.*, 1986a) with the DFP-binding proteins in normal lymphocytes. Unstimulated lymph node cells contain no detectable granzyme B (Fig. 4b) (even after exposure of the film during 2 months, data not shown), whereas a protein with the same apparent molecular weight as granzyme B is strongly labelled in lysates of the cells harvested after 6 days of stimulation. No band corresponding to the DFP-reactive 35,000 MW subunit of granzyme A present in B6.1 can be detected in lysates of the activated normal T cells (Fig. 4c). This is almost certainly due to the fact that the autoradiographic procedure is not sensible enough to detect granzyme A in normal cells which have a 100-fold less enzymatic activity than the contol CTL-cell line.

DISCUSSION

The results presented in this report show that perforin and the serine esterases granzyme A, B and C, which are not detectable in normal, resting T cells, appear as a result of the activation of pCTL by stimulation with leukoagglutinin and recombinant IL-2, i.e. under conditions which have been shown previously to be the minimal requirements for the activation of normal Lyt-2$^+$ pCTL (Erard *et al.*, 1985a, b). In contrast to the previous study, the responder cells were not purified in the experiments described here, because it was impossible to purify sufficient numbers of cells for the different assays. Thus we cannot rule out that the appearance of some of the markers assayed was influenced by cells other than the Lyt-2$^+$ T cells which constituted more than 95% of the activated populations on Day 6. Comparison of our work with that of others (Hardt, Sato & Wagner, 1987) indeed suggests that the requirements for pCTL activation may be influenced by the cell preparation methods and culture conditions.

In the course of activation with LA and IL-2, the different activities and the molecules associated with them become first detectable on Day 3. Their levels reached a maximum on Day 6 and declined on Day 7 (Fig. 2), although the cells were still growing exponentially at this time. These findings fit well with previous reports concerning the time–course of cytolytic activity in this type of culture system (Erard *et al.*, 1985a). The decline in cytolytic activity between Days 6 and 7 observed in this system correlates with a decrease in perforin and granzyme A activity. At present we do not know what controls this decrease. The observation that the percentage of cells stained with anti-perforin and anti-granzyme C antibodies also decreased suggests that the decline in haemolytic and granzyme A activities reflects a reduction in the cellular protein content rather than the appearance of inhibitors of these activities. The amounts of cytolytic activity, perforin and granzyme A contents on Day 6 in mitogen-stimulated cells (maximum of all the activities) are comparable to those found in alloantigen-stimulated cells (Garcia-Sanz *et al.*, 1987).

In general, we found a very good correlation between the relative changes in the levels of cytolytic, perforin and granzyme A activities (Fig. 2), and in all experiments they peaked on Day 6. But in some experiments the different activities became detectable on different days (Fig. 2b).

A comparison of the kinetics of appearance of perforin and granzyme A activity with that of cells containing cytoplasmic granules stained with the anti-perforin and anti-granzyme C antibodies shows that most cells already contain such granules before the population displays its peak activity levels.

Our finding that in normal activated T cells perforin activity appears with very similar kinetics as cytolytic activity is consistent with the large amount of circumstantial evidence implicating perforin as the molecule responsible for target cell lysis (Masson & Tschopp, 1985; Podack *et al.*, 1985). Our

observations are in agreement with a recent report on the appearance of perforin detected by a specific antiserum in human blood lymphocytes during activation with anti-T3 mAb (Martin *et al.*, 1987).

The role of the other molecular species detected by the assays employed here is, at present, quite unclear. The serine proteases granzyme A, B and C are contained in granules with the same density as those containing perforin (Masson *et al.*, 1986a; Pasternack *et al.*, 1986), but it has not been shown that all the molecules are contained in the same granules. Granzyme A is secreted in response to antigenic stimulation (Pasternack *et al.*, 1986; Young *et al.*, 1986b; Velotti *et al.*, 1987), and circumstantial evidence suggests that the same is true for perforin (Young *et al.*, 1986a), but it is not clear whether secretion of these or other granule constituents is regulated via a single, common pathway. We have reported that activated L3T4$^+$ cells contain no detectable perforin and very little cytolytic activity (Garcia-Sanz *et al.*,1987), and similar results have been obtained with human lymphocytes activated by stimulation with anti-T3 mAb (Martin *et al.*, 1987). But we found that L3T4$^+$ cells contain as much granzyme A as activated Lyt-2$^+$ cells. The enzyme is localized in cytoplasmic granules and secreted in response to antigen by both cell types (Garcia-Sanz *et al.*, 1987; Vellotti *et al.*, 1987). These observations clearly suggest that granzyme A is not a specific part of the lytic machinery of CTL.

The experiments described here add little information concerning the function of granzyme A, B and C, but they do suggest that common regulatory mechanisms control their synthesis. The same mechanisms may control perforin synthesis in those cells (Lyt-2$^+$) in which the perforin gene can be expressed. The only published information concerning the levels at which such regulation occurs shows that granzyme A and C mRNAs are not detectable in resting normal T cells but appear as a result of mitogen or alloantigen stimulation (Gershenfeld & Weissman, 1986; Lobe, Havele & Bleackey, 1986; Garcia-Sanz *et al.*, 1987). Correlated appearance of granzyme A, B and C, and perforin has also been observed in a T-cell hybrid in which cytolytic activity can be induced by defined stimuli (Masson *et al.*, 1985; 1986a; Brunet *et al.*, 1986).

ACKNOWLEDGMENTS

We thank Ms Françoise Bezençon and Ms Marie-Claire Combe for excellent tenchical assistance, Ms Magaly Schreyer for advice with the immunocytochemical techniques, Dr Geert Plaetinck for helpful discussions, Biogen (Geneva) for generous gift of recombinant IL-2, Mr Pierre Dubied for preparation of the illustrations and Ms Claudine Ravussin for her help in the preparation of the manuscript.

This work was supported in part by grants from the Swiss National Science Foundation and the Swiss Cancer League (to M. Nabholz and J. Tschopp). J. A. Garcia-Sanz received a grant from the Servicio de Formación de Personal Investigator (Ministerio de Educación y Ciencia), Spain.

REFERENCES

Bevan M.J., Langman R.E. & Cohn M. (1976) H-2 antigen-specific cytotoxic T cells induced by concanavalin A: estimation of their relative frequency. *Eur. J. Immunol.* **6,** 150.

Brunet J.F., Dosseto M., Denizot F., Mattei M.G., Clark W.R., Haqqi T.M., Ferrier P., Nabholz M., Schmitt-Verhulst A.M., Luciani M.F. & Golstein P. (1986) The inducible cytotoxic T-lymphocyte-associated gene transcipt CTLA-1 sequence and gene localization to mouse chromosome 14. *Nature (Lond.)*, **322,** 268.

Devos R., Plaetinck G., Cheroutre H., Simons G., Degrave W., Tavernier J. Remaut E. & Fiers W. (1983) Molecular cloning of human interleukin 2 cDNA and its expression in *E. coli. Nucl. Acids Res.* **11,** 4307.

Erard F., Corthésy P., Nabholz M., Lowenthal J.W., Zaech P. Plaetinck G. & MacDonald H.R. (1985a) Interleukin 2 is both necessary and sufficient for the growth and differentiation of lectin-stimulated cytolytic T lymphocyte precursors. *J. Immunol.* **134,** 1644.

Erard F., Nabholz M., Dupuy-d'Angeac A. & MacDonald H.R. (1985b) Differential requirements for the induction of interleukin 2 responsiveness in L3T4$^+$ and Lyt-2$^+$ T cells subsets. *J. exp. Med.* **162,** 1738.

Erard F., Nabholz M. & MacDonald H.R. (1985c) Antigen stimulation of cytolytic T lymphocyte precursors: minimal requirements for growth and acquisition of cytolytic activity. *Eur. J. Immunol.* **15,** 798.

Garcia-Sanz J.A., Plaetinck G., Velotti F., Masson D., Tschopp J., MacDonald H.R. & Nabholz M. (1987) Perforin is present only in normal activated Lyt 2$^+$ T lymphocytes and not in L3T4$^+$ cells, but the serine protease granzyme A is made by both subsets. *EMBO J.* **6,** 933.

Gershenfeld H.K. & Weissman I.L. (1986) Cloning of a cDNA for a T cell-specific serine protease from a cytotoxic T lymphocyte. *Science*, **232,** 854.

Guesdon J.P., Ternynck T. & Avrameas S. (1979) The use of avidin-biotin interaction in immunoenzymatic techniques. *J. Histochem. Cytochem.* **27,** 1131.

Hardt C., Sato N. & Wagner H. (1987) Functional and biochemical characteristics of a murine interleukin 2 receptor-inducing factor. *Eur. J. Immunol.* **17,** 209.

Henkart P.A. (1985) Mechanism of lymphocyte-mediated cytotoxicity. *Ann. Rev. Immunol.* **3,** 31.

Jenne D., Rey C., Masson D., Stanley K.K., Plaetinck G. & Tschopp J. (1988) cDNA cloning of granzyme C, a granule-associated serine protease of cytolytic T-lymphocytes. *J. Immunol.* **140,** 318.

Kramer M.D., Binninger L., Schirrmacher V., Moll H., Prester M., Nerz G. & Simon M.M. (1986) Characterization and isolation of a trypsin-like serine protease from a long-term culture cytolytic T cell line and its expression by functionally distinct T cells. *J. Immunol.* **136,** 4644.

Laemmli U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.)*, **227,** 680.

Lobe C.G., Havele C. & Bleackey R.C. (1986) Cloning of two genes that are specifically expressed in activated cytotoxic T lymphocytes. *Proc. natl. Acad. Sci. U.S.A.* **83,** 1448.

Martin D.E., Zalman L.S., Jung G. & Muller-Eberhard H.J. (1987) Induction of synthesis of the cytolytic C9 (ninth component of complement)-related protein in human peripheral mononuclear cells by monoclonal antibody OKT3 or interleukin 2: correlation with cytotoxicity and lymphocyte phenotype. *Proc. natl. Acad. Sci. U.S.A* **84,** 2946.

Masson D., Corthesy P., Nabholz M. & Tschopp J. (1985) Appearance of cytolytic granules upon induction of cytolytic activity in CTL-hybrids. *EMBO J.* **4,** 2533.

Masson D., Nabholz M., Estrade C. & Tschopp J. (1986a) Granules of cytolytic T-lymphocytes contain two serine-esterases. *EMBO J.* **5,** 1595.

Masson D. & Tschopp J. (1985) Isolation of a lytic, pore-forming protein (perforin) from cytolytic T-lymphocytes. *J. biol. Chem.* **260,** 9069.

Masson D. & Tschopp J. (1987) A family of serine esterases in lytic granules of cytolytic T-lymphocytes. *Cell*, **49,** 685.

Masson D., Zamai M. & Tschopp J. (1986b) Identification of granzyme A isolated from cytotoxic T-lymphocyte-granules as one of the proteases encoded by CTL-specific genes. *FEBS*, **208,** 84.

Nabholz M. & MacDonald H.R. (1983) Cytolytic T lymphocytes. *Ann. Rev. Immunol.* **1,** 273.

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

PASTERNACK M.S., VERRET C.R., LIU M.A. & EISSEN H.N. (1986) Serine esterase in cytolytic T lymphocytes. *Nature (Lond.)*, **322**, 740.

PODACK E.R. & KONIGSBERG P.J. (1984) Cytolytic T cell granules. *J. exp. Med.* **160**, 695.

PODACK E.R., YOUNG J.D.E. & COHN Z.A. (1985) Isolation and biochemical and functional characterization of perforin 1 from cytolytic T-cell granules. *Proc. natl. Acad. Sci. U.S.A.* **82**, 8629.

VELOTTI F., MACDONALD H.R. & NABHOLZ M. (1987) Granzyme A secretion by normal activated LyT2$^+$ and L3T4$^+$ T cells in response to antigenic stimulation. *Eur. J. Immunol.* **17**, 1095.

YOUNG J.D.E., LEONG L.G., LIU C-C., DAMIANO A. & COHN Z.A. (1986a) Extracellular release of lymphocyte cytolytic pore-forming protein (perforin) after ionophore stimulation. *Proc. natl. Acad. Sci. U.S.A.* **83**, 5668.

YOUNG J.D.E., LEONG L.G., LIU C.-C., DAMIANO A., WALL D.A. & COHN Z.A. (1986b) Isolation and characterization of a serine esterase from cytolytic T cell granules. *Cell*, **47**, 183.