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Comparative study of *in vitro* and *in vivo* drug effects on cell-mediated cytotoxicity

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Summary. Cell-mediated cytolysis (CMC) was assayed in a system using spleen cells from mice (C57BL/6) sensitized with allogeneic tumour cells (DBA/2 mastocytoma P-815). Anti-inflammatory drugs, immunosuppressives, inhibitors of cell division and other agents were investigated for their capacity to inhibit CMC in three different ways. First, inhibition of CMC after *in vitro* addition of drug was observed with corticosteroids, some immunosuppressives and inhibitors of cell division. Secondly, suppression of CMC after a single drug administration to sensitized mice shortly before being killed was found with corticosteroids, several immunosuppressives and irradiation. Thirdly, prevention of development of CMC by repeated drug treatment (immunosuppressive schedule) was achieved with most immunosuppressives and cytostatic drugs. Non-steroidal anti-inflammatory drugs were inactive in these tests. Correlation of effects between the three procedures was very poor and it is suggested that various mechanisms may be involved in the different assays.

INTRODUCTION

Cell-mediated immunity is of probable significance in certain autoimmune diseases and in chronic inflammation. If this is the case, then a search for drugs able to either interact specifically with sensitized effector cells or to prevent sensitization of immunocompetent cells constitutes a prerequisite for a therapeutic approach.

In the present experiments a series of known anti-inflammatory, immunosuppressive and anti-mitotic substances were investigated in three different ways for their ability to affect lymphocyte-mediated cytolysis. Two recently discovered compounds exhibiting immunosuppressive properties were also included, i.e. the novel anti-lymphocytic agent cyclosporin A (Rüegger, Kuhn, Lichti, Loosli, Huguenin, Quiquerez & von Wartburg, 1976; Borel, Feurer, Gubler & Stähelin, 1976) and *O*-nicotinoyl-neoconiothyrin, the derivative of a fungus metabolite isolated from an unspecified species of *Coniothyrium* (unpublished results). Using the standard method for cell-mediated target cell lysis developed by Brunner, Mauel, Cerottini & Chapuis (1968), the inhibitory activity on presensitized lymphocytes was evaluated after addition of the drug *in vitro*. In another approach the *in vivo* effect on presensitised lymphocytes was measured by administering the drug to the sensitized mice shortly before the assay. Finally, to assess the immunosuppressive capacity, i.e. to prevent sensitization of immunocompetent cells *in vivo*, drugs were given repeatedly to animals, shortly before and/or after inoculation of the target cells.

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Table 1. List of substances tested

Substances tested	Diluting agents*	Mechanism of action
Anti-inflammatory drugs		
Prednisolone (Sigma)	1/2	Lympholytic agent
Hydrocortisone succinate (Upjohn)	3	Lympholytic agent
Hydrocortisone acetate (Fluka)	2	Lympholytic agent
Dexamethasone (Ciba-Geigy)	4	Anti-inflammatory agent
Indomethacin (Merck)	4	Anti-inflammatory agent
Phenylbutazone (Ciba-Geigy)	5	Anti-inflammatory agent
Naproxen (Syntex)	5	Anti-inflammatory agent
Sodium salicylate (Merck)	3	Anti-inflammatory agent
Cytostatic and/or immunosuppressive drugs		
Cyclophosphamide (Asta)	3	Alkylation
Procarbazine (Roche)	3	Alkylation, oxidation
Cytosine arabinoside (Nutritional Biochem. Co)	3	Pyrimidine analogue
Azathioprine (Wellcome)	6/2	Purine analogue
Amethopterin (Lederle)	6/2	Folic acid antagonist
Cyclosporin A (Sandoz)	7/2	Unknown
O-Nicotinoyl-neoconiothyrin (Sandoz)	4	Unknown
Antilymphocyte serum (ALS) (Sandoz)	3	Lymphocytotoxic antibodies
Inhibitors of cell division		
Demecolcine (Ciba-Geigy)	3	Spindle poison
Colchicine (Sandoz)	3	Spindle poison
Vincristine (Lilly)	3	Spindle poison
Epipodophyllotoxin derivative VP 16-213 (Sandoz)	4	Mitotic inhibitor (G2 blocker)
Cytochalasin B (Sandoz)	8	Inhibition of microfilament contractility
Miscellaneous agents		
Trypsin (Difco)	9	Endopeptidase
Di-potassium ethylenediamine-tetraacetate (Fluka)	3	Chelating agent
Adenosine-3′,5′cyclic mono-phosphate (Fluka)	3	Secondary messenger

* (1) 0·05 ml ethanol for 1 mg substance; (2) 0·5 per cent tragacanth (suspension for *in vivo* administration); (3) Hanks's solution; (4) 0·1 ml DMSO (dimethylsulphoxide) plus 0·02 ml Tween 80 for 1 mg substance; (5) 0·1 ml 5 per cent sodium bicarbonate for 1 mg substance; (6) 0·05 ml 2·5 per cent sodium bicarbonate for 1 mg substance; (7) 0·3 ml ethanol plus 0·025 ml Tween 80 for 1 mg substance; (8) 1·0 ml DMSO for 1 mg substance; (9) phosphate-buffered saline, pH 7·4.

MATERIALS AND METHODS

Drugs

The generic names of the substances tested and their source are listed in Table 1.

In vitro *cytotoxicity assay with sensitized lymphoid cells*

The cytotoxic action of specifically sensitized lymphocytes on ^{51}Cr-labelled allogeneic target cells was measured according to the method of Brunner *et al.* (1968). In addition to some minor alterations a few important details have to be specified. Spleens from C57BL/6 mice were harvested 10–12 days following a single intraperitoneal immunizing dose of 30–60 × 10^6 DBA/2 mastocytoma P-815-X2 cells. The spleen cell suspension was allowed to sediment for 10 min at 4°. Determination of viable spleen cells (but not of target cells) using the Evans blue exclusion test was usually omitted. The labelling of target cells was performed with sodium chromate ([^{51}Cr]-sodium chromate, specific activity 100–300 mCi/mg

of chromium) (Radiochemical Centre, Amersham, Bucks.), 40 μCi/0·4 ml containing 2×10^6 target cells, but the incubation time was prolonged to 45 min. The ratio of sensitized spleen cells to target cells was invariably 100:1. The incubation period of the reaction mixture was limited to 4·5 h throughout these experiments. The reaction mixture consisted of equal volumes (0·2 ml) of test substance diluted in 10^{-2} M Tris buffer at pH 7·4, of spleen cell and pre-labelled target cell suspensions. The test substance was added first into the tubes, then the lymphocyte suspension and lastly the ^{51}Cr-labelled target cells. The final concentration in the whole reaction mixture is indicated in Table 2. All controls and drug samples were tested in triplicate. The percentage of ^{51}Cr release is calculated for each sample as described by Brunner *et al.* (1968). The term 'inhibition' is used to describe the anticytolytic or protective effect of drugs for the target cell. Percentage inhibition is determined on the basis of difference in label release between reaction mixtures containing sensitized lymphoid cells and drug as compared to the same mixtures in the absence of drug.

Late treatment of sensitized mice with a single drug injection

Mice were sensitized with allogeneic target cells and the cytolytic assay was performed as described above, but no drug was added *in vitro*, the corresponding amount being replaced by 10^{-2} M Tris buffer (pH 7·4). Instead, a single dose of drug was administered intraperitoneally, orally or subcutaneously to groups of sensitized mice 16 h before sacrifice, i.e. on the 9th to 11th day after immunization. Only non-lethal doses of drug were injected (mortality <20 per cent within 10 days). Each group consisted of five to six mice, whose spleens were processed individually. The percentage of inhibition was calculated on the basis of difference in label release between reaction mixtures containing lymphoid cells from drug-treated sensitized mice as compared to the same mixtures containing lymphoid cells from untreated sensitized control mice. Only the means of the groups were compared; at least three groups per substance were tested in separate experiments, thus producing fifteen or more individual results. From these, reliability and reproducibility of the drug effect was adequately evaluated. Furthermore, viability of suspended spleen cells was determined by Evans blue dye exclusion for each drug. Since percentage of dead cells in treated cultures did not exceed that of control cultures ($9·0 \pm 5·1$ per cent (s.d.); $n = 70$) by more than 1 s.d., it seems reasonable to consider the observed inhibition of target cell destruction as drug-dependent and not just due to damaged effector cells unable to exert their cytolytic function.

Early treatment of sensitized mice by repeated drug administration

Groups of five to six C57BL/6 ♀ adult mice were sensitized with allogeneic target cells as previously described and immediately subjected to an intensive immunosuppressive treatment. To attain optimal depression of sensitized lymphocytes several treatment schedules (dose, route, days) were required depending on the drug used. Therefore the schedule for each drug is indicated in Table 4. Although satisfactory results were sometimes obtained with other variations of treatment they are not considered here. All drugs were used in doses killing <20 per cent of the animals during the experiment. Control mice received intraperitoneal injections of diluting agent. The animals were killed 11–12 days after immunization and their spleens assessed individually for the presence of cytolytic effector cells. The percentages of immunological suppression of the means of groups were compared.

RESULTS

In vitro induced drug effects on cell-mediated cytotoxicity

Although the highest concentrations mentioned in Table 2 may not always be those attainable *in vivo*, they were never cytotoxic as ascertained by two tests. ^{51}Cr release by labelled spleen cells incubated with any of the substances never exceeded 10 per cent of the value obtained with control cells incubated with medium alone. In the dye exclusion test using Evans blue the differences remain within the limit of 10 per cent of control values. These cytotoxic tests were performed over a period of 4·5 h which corresponds to the incubation time used for *in vitro* cell-mediated cytolysis. Solvents alone were also included in the test, but they did not affect the cytotoxic activity of immune spleen cells. The final test dilution of dimethyl sulphoxide never exceeded 0·25 per cent, which was non-toxic. DMSO in higher concentrations is known to reversibly inhibit specific cytolysis (Wolberg, Hiemstra, Burge & Singler, 1973).

Table 2. *In vitro* effect of various substances on the cytolytic interaction of sensitized lymphocytes and ^{51}Cr-labelled allogeneic target cells

Substances tested	Final concentration	Percentage reduction of ^{51}Cr release		Final concentration	Percentage reduction of ^{51}Cr release	
	μg/ml	Mean ± s.e.m.		μg/ml	Mean ± s.e.m.	
Anti-inflammatory drugs						
Prednisolone	100	68·5 ± 6·8	(4)*	10	7·8 ± 3·9	(4)*
Hydrocortisone succinate	1000	80·0 ± 4·6	(3)	100	21·0 ± 2·3	(5)
Dexamethasone	10	5·5 ± 1·8	(4)	1	6·3 ± 2·3	(3)
Indomethacin	10	24·8 ± 7·1	(4)	1	1·0 ± 0·6	(4)
Phenylbutazone	100	18·7 ± 3·5	(6)	10	1·5 ± 1·5	(4)
Naproxen	100	9·7 ± 4·8	(3)	10	4·5 ± 3·3	(4)
Sodium salicylate	1000	14·3 ± 2·2	(3)	100	5·7 ± 3·2	(3)
Immunosuppressives						
Cyclophosphamide	100	1·0 ± 1·0	(3)	10	1·3 ± 0·9	(3)
Procarbazine	100	2·0 ± 1·5	(3)	10	2·0 ± 1·5	(3)
Cytosine arabinoside	10	0·5 ± 0·5	(4)	1	0·8 ± 0·5	(4)
Azathioprine	100	53·3 ± 8·2	(4)	10	4·3 ± 2·5	(4)
Amethopterin	100	4·0 ± 2·6	(3)	10	12·3 ± 6·1	(4)
Cyclosporin A	10	35·7 ± 5·4	(6)	1	3·4 ± 1·5	(5)
Neoconiothyrin derivative	3	52·8 ± 3·6	(5)	1	27·6 ± 6·0	(5)
Antilymphocyte serum	1:30	91·0 ± 4·4	(8)	1:300	68·4 ± 11·2	(5)
Inhibition of cell division						
Demecolcine	10	56·0 ± 2·2	(4)	0·1	53·5 ± 6·4	(4)
Colchicine	10	46·0 ± 5·3	(6)	1	36·8 ± 4·2	(4)
Vincristine	10	60·5 ± 3·1	(6)	1	42·3 ± 3·4	(6)
Epipod. VP 16–213	10	5·6 ± 2·4	(5)	1	1·5 ± 1·5	(2)
Cytochalasin B	1	64·8 ± 5·3	(8)	0·1	16·3 ± 2·4	(6)
Miscellaneous agents						
Trypsin	2000	91·3 ± 1·5	(3)	—		
Di-potassium EDTA	2000	96·3 ± 2·0	(3)	400	5·0 ± 2·9	(3)
Cyclic AMP	10	40·2 ± 7·0	(6)	1	14·7 ± 2·0	(3)

Substances are added *in vitro* to a suspension of sensitized C57BL/6 mouse spleen cells; thereafter ^{51}Cr-labelled mastocytoma cells (P-815-X_2) from DBA/2 mice are added. Inhibition of specific ^{51}Cr release by target cells during 4·5 h incubation is expressed in percentage of controls.

* Number of experiments.

Among the anti-inflammatory drugs, prednisolone and hydrocortisone produce a definite reduction of ^{51}Cr release, but this effect occurs only at relatively high concentrations. Indomethacin and phenylbutazone show a marginal effect at 10 and 100 μg/ml, respectively. The inhibition rates of dexamethasone, naproxen and sodium salicylate are too low to be considered to differ from the controls. Four of the cytostatic and/or immunosuppressive compounds (ALS, neoconiothyrin, azathioprine and cyclosporin A) inhibit cell-mediated cytolysis *in vitro*. Procarbazine, cytosine arabinoside, amethopterin and cyclophosphamide, which latter drug has to be metabolized *in vivo* to become active, remain ineffective. Cytochalasin B, vincristine, demecolcine and colchicine which are drugs known to interfere with cell division are potent inhibitors of cell-mediated cytotoxicity. Epipodophyllotoxin derivative VP 16-213, however, exhibits no such activity. The miscellaneous agents dipotassium EDTA, trypsin and cyclic AMP markedly reduce ^{51}Cr release by target cells.

The inhibitory activity of most substances was measured at several concentrations. Demecolcine exerts its activity over a concentration range from 10 to 0·1 μg/ml. In contrast, the marked effect of prednisolone at 100 μg/ml or of cytochalasin B at 1 μg/ml disappears in a ten-fold lower concentration.

Table 3. Inhibition of target cell lysis after *in vivo* treatment with drug of sensitized mice 9–11 days post-immunization

Substances tested	Dose (mg/kg)	Percentage inhibition Mean ± s.e.m.	Mice (dead/total)
Anti-inflammatory drugs			
Prednisolone	100†	61·3 ± 5·6	0/18
Hydrocortisone acetate	300*	94·0 ± 2·0	0/15
Dexamethasone	5	72·7 ± 6·1	0/15
Phenylbutazone	200	6·8 ± 2·1	0/22
Immunosuppressives			
Cyclophosphamide	300	69·3 ± 4·2	0/20
Procarbazine	300	47·5 ± 2·4	0/20
Cytosine arabinoside	100	0·6 ± 0·4	0/27
Azathioprine	500	71·3 ± 3·8	0/14
Amethopterin	150	59·3 ± 1·5	0/17
Cyclosporin A	500†	8·8 ± 2·6	0/19
Neoconiothyrin derivatives	50	68·0 ± 6·0	0/21
Anti-lymphocyte serum	0·2 ml	59·2 ± 7·2	1/24
Inhibition of cell division			
Colchicine	1	3·6 ± 2·9	0/24
Vincristine	1	11·4 ± 3·0	0/26
Epipod. VP 16–213	50	66·0 ± 1·2	0/15
Irradiation	450 r	53·0 ± 3·2	0/22

All mice received either one dose of substance or the equivalent amount of diluting agent 16 h before being killed. Groups consisted of five to six C57BL ♀ mice. Each spleen was assessed separately for cytolytic activity of sensitized lymphocytes.

* Subcutaneous and †oral administration; otherwise intraperitoneal injection.

Other compounds such as vincristine show a dose–response curve with an intermediary slope.

In vivo drug effects after late treatment of sensitized mice

Having demonstrated inhibition of the cytotoxicity of sensitized lymphocytes following admixture of certain drugs in nontoxic concentrations *in vitro*, the next step towards the possible therapeutic use of this drug effect was to investigate the ability of such substances to cause a similar inhibition after administration *in vivo* shortly before killing the animal. Sensitized mice were treated with a single dose of drug 16 h before being killed and their spleen cells were assayed *in vitro* as described above, but without any further addition of drug to the cultures. The period of 16 h between drug administration and cell harvest was chosen for practical reasons (see also Discussion).

The results obtained *in vivo* with many of the substances previously assessed *in vitro* are summarized in Table 3. Several drugs which were strongly active *in vitro* have also shown a clear inhibition after *in vivo* administration: prednisolone, hydrocortisone, azathioprine, neoconiothyrin, and ALS. Other agents which showed little (phenylbutazone) or no activity *in vitro* (cytosine arabinoside) remained equally ineffective following *in vivo* treatment. Some drugs, however, which lacked any inhibitory effect *in vitro*, turned out to be highly active *in vivo* (dexamethasone, cyclophosphamide, procarbazine, amethopterin and VP 16-213), or the reverse situation occurred, i.e. effect *in vitro* and loss of activity *in vivo* (colchicine, vincristine and cyclosporin A). Sublethal irradiation severely affected the cytolytic function of effector cells. Other results obtained with some of these compounds when further tested in different doses or given by another route or at a different time before the assay were not included in this table. It was found that the neoconiothyrin derivative lost its activity when administered orally (300 mg/kg) and that 0·5 ml of ALS resulted in a total inhibition without major toxic effects. Repeated administration of a few ineffective substances, i.e. 16 and 2 h before the assay, remained unsuccessful.

The present results reveal a marked discrepancy between the *in vitro* and *in vivo* activity of a drug,

Table 4. Immunosuppression of cell-mediated cytotoxicity due to drug treatment of mice at onset of sensitization

Substances tested	Dose (mg/kg/day)	Route	Treatment on days	Percentage inhibition (mean ± s.e.m.)	Mice (dead/total)
Anti-inflammatory drugs					
Hydrocortisone acetate	40	s.c.	−3, −1, 1, 3	5·0 ± 3·6	1/22
Dexamethasone	1	i.p.	0–7	15·0 ± 3·5	0/15
Phenylbutazone	100	i.p.	1–7/1, 3, 5, 7, 9	5·0 ± 4·0	0/17
Immunosuppressives					
Cyclophosphamide	40	p.o.	0–7	98·7 ± 0·7	3/21
Procarbazine	50	p.o.	0–7/1–8	93·7 ± 3·3	0/21
Cytosine arabinoside	2 × 10	i.p.	0–7	33·7 ± 1·5	0/15
Azathioprine	50/80	p.o.	0–7	90·3 ± 1·9	4*/20
Amethopterin	1	i.p.	0–6/1, 3, 5, 7	86·3 ± 10·0	0/15
Cyclosporin A	150/200	p.o.	0–7	83·0 ± 16·7	2*/20
Neoconiothyrin deriv.	10/20	i.p.	0–6	80·0 ± 5·4	6/32
Anti-lymphocyte serum	0·2 ml	i.p.	−3 or/and −1	91·3 ± 6·7	3*/15
Inhibition of cell division					
Colchicine	0·4	i.p.	0–7	27·6 ± 5·8	2/36
Vincristine	0·2	i.p.	0–7	79·3 ± 7·7	0/20
Epipod. VP 16–213	15	i.p.	1, 3, 6	90·5 ± 2·4	1/20

Dose, route and days of drug administration are indicated for each compound. With some drugs either of the two schedules listed produced similar results. Groups of five to six adult C57BL/6 ♀ mice were used. Each experiment included one group of sham-treated control mice. Each spleen was assessed separately for cytotoxic lymphocytes, but only means of groups are considered (± s.e.m.).

* Mice died of ascites; tumour development due to immunosuppression.

thereby indicating that *in vivo* effectiveness is difficult to predict on the basis of *in vitro* results alone.

Suppression of cytotoxic lymphocytes following repeated drug administration early after sensitization

Once the inhibitory effect on sensitized spleen cells by several compounds after either *in vitro* addition or single *in vivo* treatment had been found, the search for compounds impairing the sensitization of lymphocytes by allogeneic target cells was undertaken. A modification of the previous method was adopted. Drugs were repeatedly administered immediately before and/or after injection of mastocytoma cells in order to prevent the development of sensitized lymphocytes.

As can be seen from Table 4 the anti-inflammatory drugs hydrocortisone, dexamethasone and phenylbutazone remained without influence on the development of cell-mediated cytotoxicity. However, all immunosuppressive compounds tested caused a highly reproducible suppression of lymphocyte sensitization. Cyclosporin A was the only drug further investigated for the suppression of the secondary response. The mice used in these experiments did not receive any immunosuppressive treatment at the time of the primary response and were challenged 20 weeks later by a booster injection of irradiated mastocytoma cells. The animals were treated with 300 mg/kg/day of cyclosporin A given orally on days 0, 1, 2, 4 and 5. Cytolysis was measured on days 7 and 9 and yielded 50–70 per cent inhibition. The three inhibitors of cell division when given intraperitoneally also strongly depressed the primary development of sensitized spleen cells. Therefore, an immunosuppressive regime with cytostatic or immunosuppressive agents successfully prevented the development of sensitised lymphocytes, while the anti-inflammatory drugs investigated did not exert such an effect. The latter substances act on lymphocytes by mechanisms differing from those of the other two groups.

These results should be viewed with some caution for two reasons. The schedule of treatment can be very critical and this particularly pertains to VP 16-213 (Dombernowsky & Nissen, 1973; Stähelin, 1973). It is therefore possible that some drugs were not applied in the proper way to yield noticeable

Table 5. Summary of drug effects on cell-mediated cytotoxicity obtained in three different modifications of the assay method

Drugs tested	Effects on cell-mediated cytotoxity following:		
	In vitro addition before target cells	Late *in vivo* treatment with a single dose	Early immunosuppressive treatment with several doses
Steroids			
Prednisolone	+	+	
Hydrocortisone succinate	+	+	0
Dexamethasone	0	+	0
Non-steroidal anti-inflammatory drugs			
Indomethacin	±		
Phenylbutazone	0	0	0
Naproxen	0		
Sodium salicylate	0		
Cytostatic drugs			
Cyclophosphamide	0	+	+
Procarbazine	0	+	+
Cytosine arabinoside	0	0	+
Azathioprine	+	+	+
Amethopterin	0	+	+
VP 16–213	0	+	+
Immunosuppressives			
Cyclosporin A	+	0	+
Neoconiothyrin derivative	+	+	+
Anti-lymphocyte serum	+	+	+
Mitotic inhibitors			
Demecolcine	+		
Colchicine	+	0	±
Vincristine	+	0	+
Cytochalasin B	+	0*	0*
Miscellaneous agents			
Trypsin	+		
Di-potassium EDTA	+		
Cyclic AMP	+		
Irradiation (X-rays)		+	

* Cytochalasin B is not active *in vivo* (unpublished results).

effects. As immune cytotoxicity was always assayed at the height of a normal response, a reduction of target cell lysis at this time may not necessarily reflect genuine inhibition, but could merely mask a delay in the peak response. Moreover, conclusive evidence emerges that an immunosuppressive effect on the development of cytolytic lymphocytes does not allow prediction of the effect of the same drug on already sensitised lymphoid cells either following acute *in vivo* treatment or *in vitro* addition.

Table 5 represents a synopsis of the results obtained with all drugs tested in the various modifications of the assay for cell-mediated cytolysis. It is seen that several patterns of effects occur within all classes of drugs, thus precluding any predictions.

DISCUSSION

The *in vitro* cytotoxic assay system used in this study is based on the cytolytic action of sensitised lymphoid cells against ^{51}Cr-labelled allogeneic target cells and measures release of radioactive label as a function of target cell destruction (Brunner, Mauel, Rudolf & Chapuis, 1970; Mauel, Rudolf, Chapuis & Brunner, 1970). It is now well established that the specifically cytotoxic spleen cells arising in mice immunized by tumour allografts represent thymus-derived lymphocytes and that this type of immune response does not involve bone marrow-derived cells nor antibodies and complement (reviewed by Cerottini & Brunner, 1974; Henney, 1974). Moreover, this

type of immune cytolysis depends on the recognition of transplantation antigens by specific receptors present on the surface of sensitised T lymphocytes (Bonavida, 1974). Although the differentiation and fate of cytotoxic T lymphocytes *in vitro* have recently been elucidated (MacDonald, Cerottini & Brunner, 1974), the mechanism of immune cytolysis is still poorly understood. It is generally recognized that actual contact of sensitized lymphocytes and target cell is necessary to initiate the lytic pathway. Target cell lysis occurs at a second stage and is possibly mediated by the release of cytotoxic factors from the effector cells at the site of contact (Granger & Kolb, 1968; Peter & Dawkins, 1971) and/or by a progressive breakdown of the target cell membrane integrity leading finally to osmotic lysis (Ferluga & Allison, 1974; Martz, Burakoff & Benacerraf, 1974).

Can the cytotoxic action of the effector cells be altered by *in vitro* addition of drugs? Several substances with widely differing characteristics have efficiently reduced the rate of target cell lysis once they had been added *in vitro* to the effector cells (Table 2).

The effector cell must be viable to exert its cytolytic action; this is abolished after a high irradiation dose (Rosenau & Moon, 1966) or upon treatment with antiserum and complement (Freedman, Cerottini & Brunner, 1972). Therefore the marked suppression of the cytolytic effect by prednisolone, hydrocortisone and ALS could be related to their lymphotoxic properties (depression of cellular functions before cell death), although the concentrations used did not apparently affect lymphocyte viability (^{51}Cr release and dye exclusion tests) during the incubation period of 4·5 h. Mauel *et al.* (1970) have demonstrated the suppressive effect of ALS directed against the attacking lymphoid cells. Results concerning the influence of corticosteroids on the effector phase are controversial (Rosenau and Moon, 1966; Cohen, Stavy and Feldman, 1970; Mauel *et al.*, 1970). Sanderson & Franks (1975) have recently demonstrated a short prednisolone-sensitive induction period which involves an unknown, but reversible very early action of the drug on the effector lymphocyte. Surprisingly, dexamethasone did not seem to affect sensitized spleen cells. However, these corticosteroids and ALS strongly inhibited tritiated thymidine incorporation and proliferation of concanavalin A stimulated murine lymphocytes at 1 μg/ml, respectively at 1:1000 (unpublished data). In spite of the doubtful effect shown by indomethacin and phenylbutazone, none of the non-steroidal antiphlogistic drugs investigated effectively prevented target cell damage. Winchurch, Foschi & Walz (1974) tested anti-inflammatory drugs in a similar model system in rats, but using an incubation time of 16–18 h. It is our experience that results obtained after such a long period are at great variance with those obtained during only 3–5 h. However, they reported a wide dose–response effect from 10^{-10} to 10^{-7} M with hydrocortisone, but aspirin, indomethacin and phenylbutazone were effective only over a narrow range of high concentrations (10^{-4}–10^{-3} M).

The effector cell must be metabolically active in in order to exert cytotoxicity (for review compare Cerottini & Brunner, 1974). Several substances which interfere with cell metabolism in its widest sense were investigated for their *in vitro* effects on cell-mediated cytotoxicity (Table 2). The divergent results produced by the immunosuppressive drugs which interfere with DNA synthesis do not allow any conclusion whether DNA synthesis is necessary to sustain cytolytic activity of sensitized lymphocytes. As cyclophosphamide needs to be activated *in vivo* to exert its therapeutic effect, negative results were anticipated. Mauel *et al.* (1970) did not find diminished cytotoxic activity in the presence of fluoro-deoxyuridine, an inhibitor of thymidine synthetase. The mechanisms of action of cyclosporin A and neoconiothyrin, which are microbial metabolites from fungi clearly inhibiting cytotoxicity, are unknown, although there is evidence from other experiments not presented here that they suppress cell-mediated immunity.

The mitotic inhibitor VP 16-213 failed to impair target-cell killing, but the three mitotic poisons tested, demecolcine, colchicine and vincristine, produced a marked inhibition. Cerottini & Brunner (1972) have not, but Plaut, Lichtenstein & Henney (1973) have demonstrated an inhibitory effect with colchicine in this assay system. Cytochalasin B which interacts with several cellular functions, including cell movement, reversibly affected cytotoxicity as previously reported (Borel & Stähelin, 1972). Of these five drugs (with the possible exception of epipodophyllotoxin derivative VP 16-213) three are known to disrupt microtubules or microfilaments (Wilson, 1970) and four have further been shown to inhibit chemotactic migration *in vitro* of polymorphonuclear leucocytes (Borel, 1973) as well as of macrophages (Borel & Feurer, 1975). These results are suggestive

of a possible interaction of these agents with a secretory process in the lytic pathway (Strom, Garovoy, Carpenter & Merrill, 1973; Henney, 1973) and/or of altered cell surface mobility which ultimately prevents lymphocyte–target cell binding (Ferluga, Asherson & Becker, 1972; Stulting, Berke & Hiemstra, 1973; Bubbers & Henney, 1975). In his review, Henney (1973) provides evidence for the existence of multiple stages in cell-mediated cytolysis. Colchicine appears to irreversibly suppress cytolytic expression at a late stage, while cytochalasin B reversibly inhibits at an early stage of the lytic pathway (Plaut *et al.*, 1973).

Our results showing abolition of cell-mediated cytolysis by treatment of sensitized spleen cells with trypsin are consistent with those from others (Mauel *et al.*, 1970; Werkele, Lonai & Feldman, 1972). The use of proteolytic enzymes is thought to degrade specific receptors on the membrane of the effector cell. Complete blocking of target cell lysis was also achieved with high concentrations of the chelating agent EDTA, which proves most effective when added at the onset of the incubation period (Mauel *et al.*, 1970). The absence of divalent cations may disturb cellular metabolism and also be implicated in secretory events. Henney & Bubbers (1973) have distinguished a cation-dependent phase of the lytic reaction occurring after lymphocyte–target cell interaction and preceding membrane permeability changes in the target cell. Addition of cyclic 3′,5′-adenosine monophosphate (cAMP) (10–100 μg/ml), N^6-monobutyryl 3′,5′-cAMP and N^6, O'–2′dibutyryl 3′,5′-cAMP (100 μg/ml), but not of five other related nucleotides (results not presented here), caused some reduction of cytotoxic killing. Henney, Bourne & Lichtenstein (1972) and Strom, Carpenter, Garovoy & Austen (1973) have demonstrated that enhanced intracellular cAMP levels inhibit cytolysis. This inhibition which follows the lymphocyte–target cell interaction could be mediated by interference of cAMP with the cellular secretory process.

Substances which clearly affect cytotoxicity *in vitro* by various mechanisms of action interfering at different stages of the lytic process may possibly also exert an inhibitory effect on immune lymphocytes following *in vivo* administration shortly before killing the animal, provided they are not too rapidly metabolised, reach the target organ and attain a local concentration sufficient to elicit an effect. This approach appeared particularly valuable to assess the therapeutic potential of a drug. However, reports along this line seem scarce.

All three corticosteroids studied abrogated the cytotoxic reaction of immune spleen cells when administered to sensitized mice 16 h before death (Table 3). Using different stimuli, Vischer (1972) also observed a reduced reactivity of spleen cells from mice treated with a single injection of hydrocortisone. Though it is known that the hydrocortisone-resistant spleen cells are capable of initiating a graft-versus-host reaction (Cohen, Fischbach & Claman, 1970), it seems that this type of drug induces some deleterious effects on surviving, sensitized lymphocytes thereby affecting their cytolytic capacity *in vitro*. Phenylbutazone failed to inhibit the cytotoxicity of sensitized lymphoid cells. The immunosuppressives produced divergent results. Cyclophosphamide has been shown *in vivo* to deplete thymus-independent, but not thymus-dependent areas in the spleen after 16 h (Stockman, Heim, South & Trentin, 1973). It is therefore surprising to observe that this drug became very inhibitory for T cells. Procarbazine and amethopterine gave positive results, while cyclosporin A remained negative following *in vivo* administration. The results with the other compounds of this group correlated well with those obtained *in vitro*. Among the inhibitors of cell division the epipodophyllotoxin derivative affected cytolytic immune lymphocytes, but colchicine and vincristine were inactive. All these compounds were administered in non-toxic, though high doses and many of them elicited powerful and long-lasting effects. For comparison, sublethal irradiation (450 r) also resulted in strong inhibition of the cytolytic effector cells. It has not yet been established whether the underlying mechanism of this inhibition observed with several drugs is similar to that of an irradiating effect or whether different mechanisms are involved.

The last part of this study deals with the prevention of the development of sensitized splenic lymphocytes. Substances were administered repeatedly to mice at the time of immunization with allogeneic tumour cells. This type of experimentation evaluates mainly the immunosuppressive activity of drugs. The results presented in Table 4 confirmed this assumption. All agents listed as cytostatic and/or immunosuppressive drugs as well as the inhibitors of cell division (possibly except colchicine) clearly depressed the development of cytotoxic lymphoid cells. Most of these agents are well-known to inhibit

the initiation of both humoral and cell-mediated immunity. Based on several experimental models not reported here, definite immunosuppressive properties could be attributed to cyclosporin A, *O*-nicotinoyl-neoconiothyrin and VP 16-213. The finding that hydrocortisone-resistant spleen cells are capable of initiating a cell-mediated immune response (Cohen *et al.*, 1970) is compatible with the failure of hydrocortisone to suppress lymphocyte sensitization. Phenylbutazone does not possess immunosuppressive properties and accordingly did not prevent development of cytolytic effector cells.

Several conclusions are emerging from this study. The assay system chosen allowed a quantitative measurement of T cell-mediated cytotoxicity. The *in vitro* addition immediately before (or shortly after) mixing effector cells with target cells of certain substances which will or will not influence immune cytolysis represents a helpful approach for elucidating underlying mechanisms of the lytic pathway. However, large-scale screening with widely differing chemicals yielded very few compounds producing interesting *in vitro* results (unpublished data). If the substances are administered once to sensitized mice at peak response several hours before being killed, many of them show differences from the *in vitro* effect. This obvious absence of correlation (see Table 5) possibly reveals the involvement of distinct mechanism of the inhibitory function. As we were primarily interested in the search for drugs capable of affecting sensitized lymphoid cells in a therapeutically useful manner, it appears that the second type of approach was more promising for this purpose. Furthermore, the inhibitory effect of drugs on the cytolytic expression of sensitized lymphocytes did apparently not correlate with cytostatic, immunosuppressive, anti-inflammatory or other defined properties. Applying an immunosuppressive schedule to prevent development of specifically sensitized spleen cells, it was shown that cytostatic agents and immunosuppressives, but not anti-inflammatory drugs, inhibited cell-mediated cytotoxicity. This property, shared by agents known to suppress sensitization of immunocompetent cells in other models of cellular immunity as well, did not correlate with the capacity to affect specifically sensitized lymphoid cells. It is, therefore, suggested that each modification of the drug–lymphocyte interaction attempted in these investigations represents a separate pharmacological entity.

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REFERENCES

BONAVIDA B. (1974) Studies on the induction and expression of T cell-mediated immunity. *J. Immunol.* **112,** 926.

BOREL J.F. (1973) Effect of some drugs on the chemotaxis of rabbit neutrophils *in vitro*. *Experientia (Basel)*, **29,** 676.

BOREL J.F. & FEURER C. (1975) Chemotaxis of rabbit macrophages *in vitro*. Inhibition by drugs. *Experientia (Basel)*, **31,** 1437.

BOREL J.F., FEURER C., GUBLER H.U. & STÄHELIN H. (1976) Biological effects of cyclosporin A: a new antilymphocytic agent. *Agents Actions*, **9,** 432.

BOREL J.F. & STÄHELIN H. (1972) Effects of cytochalasin B on chemotaxis and immune reactions. *Experientia (Basel)*, **28,** 745.

BRUNNER K.T., MAUEL J., CEROTTINI J.-C. & CHAPUIS B. (1968) Quantitative assay of the lytic action of immune lymphoid cells on ^{51}Cr-labelled allogeneic target cells *in vitro*; inhibition by isoantibody and by drugs. *Immunology*, **14,** 181.

BRUNNER K.T., MAUEL J., RUDOLF H. & CHAPUIS B. (1970) Studies of allograft immunity in mice. *Immunology*, **18,** 501.

BUBBERS J.E. & HENNEY C.S. (1975) Studies on the mechanism of lymphocyte-mediated cytolysis. *J. Immunol.* **115,** 145.

CEROTTINI J.C. & BRUNNER K.T. (1972) Reversible inhibition of lymphocyte-mediated cytotoxicity by cytochalasin B. *Nature: New Biology*, **237,** 272.

CEROTTINI J.C. & BRUNNER K.T. (1974) Cell-mediated cytotoxicity, allograft rejection, and tumor immunity. *Advanc. Immunol.* **18,** 67.

COHEN J.J., FISCHBACH M. & CLAMAN H.N. (1970) Hydrocortisone resistance of graft *vs* host activity in mouse thymus, spleen and bone marrow. *J. Immunol.* **105,** 1146.

COHEN I.R., STAVY L., & FELDMAN M. (1970). Glucocorticoids and cellular immunity in vitro. Facilitation of the sensitization phase and inhibition of the effector phase of a lymphocyte anti-fibroblast reaction. *J. exp. Med.* **132,** 1055.

DOMBERNOWSKY P. & NISSEN N.I. (1973) Schedule dependency of the antileukemic activity of the podophyllotoxin-derivative VP 16–213 in L1210 leukemia. *Acta path. microbiol. scand.* **81,** 715.

FERLUGA J. & ALLISON A.C. (1974) Observations on the mechanism by which T lymphocytes exert cytotoxic effects. *Nature (Lond.)*, **250,** 673.

FERLUGA J., ASHERSON G.L. & BECKER E.L. (1972) The effect

of organophosphorus inhibitors, p-nitrophenol and cytochalasin B on cytotoxic killing of tumour cells by immune spleen cells, and the effect of shaking. *Immunology*, **23**, 577.

FREEDMAN L.R., CEROTTINI J.C. & BRUNNER K.T. (1972) *In vivo* studies of the role of cytotoxic T cells in tumor allograft immunity. *J. Immunol.* **109**, 1371.

GRANGER G.A. & KOLB W.P. (1968) Lymphocyte *in vitro* cytotoxicity: mechanisms of immune and non-immune small lymphocyte mediated target L cell destruction. *J. Immunol.* **101**, 111.

HENNEY C.S. (1973) On the mechanism of T cell mediated cytolysis. *Transplant. Rev.* **17**, 37.

HENNEY C.S. (1974) Mechanism of cytolysis by thymus-derived lymphocytes. *Immunology of Cancer, Progress. experimental Tumor Research*, volume 19, p. 203. Karger, Basel.

HENNEY C.S., BOURNE H.R. & LICHTENSTEIN L.M. (1972) The role of cyclic 3'5'-adenosine monophosphate in the specific cytolytic activity of lymphocytes. *J. Immunol.* **108**, 1526.

HENNEY C.S. & BUBBERS J.E. (1973) Studies on the mechanism of lymphocyte-mediated cytolysis. *J. Immunol.* **110**, 63.

MACDONALD H.R., CEROTTINI J.C. & BRUNNER K.T. (1974) Generation of cytotoxic T lymphocytes *in vitro*. *J. exp. Med.* **140**, 1511.

MARTZ E., BURAKOFF S.J. & BENACERRAF B. (1974) Interruption of the sequential release of small and large molecules from tumor cells by low temperature during cytolysis mediated by T cells or complement. *Proc. nat. Acad. Sci. (Wash.)*, **71**, 177.

MAUEL J., RUDOLF H., CHAPUIS B. & BRUNNER K.T. (1970) Studies on allograft immunity in mice. *Immunology*, **18**, 517.

PETER J.B. & DAWKINS R.L. (1971) Target cell lysis mediated by soluble cytotoxin released from stimulated lymphocytes. *Nature: New Biology*, **232**, 79.

PLAUT M., LICHTENSTEIN L.M. & HENNEY C.S. (1973) Studies on the mechanism of lymphocyte-mediated cytolysis. *J. Immunol.* **110**, 771.

SANDERSON C.J. & FRANKS D. (1975) Effect of prednisolone on cell-mediated cytotoxicity *in vitro*. *Int. Arch. Allergy*, **48**, 610.

STÄHELIN H. (1973) Activity of a new glycosidic lignan derivative (VP 16–213) related to podophyllotoxin in experimental tumors. *Europ. J. Cancer*, **9**, 215.

STOCKMAN G.D., HEIM L.R., SOUTH M.A. & TRENTIN J.J. (1973) Differential effects of cyclophosphamide on the B and T cell compartments of adult mice. *J. Immunol.* **110**, 277.

STROM T.B., GAROVOY M.R., CARPENTER C.B. & MERRILL J.P. (1973) Microtubule function in immune and non-immune lymphocyte-mediated cytotoxicity. *Science*, **181**, 171.

STROM T.B., CARPENTER C.B., GAROVOY M.R., AUSTEN K.F., MERRILL J.P. & KALINER M. (1973) The modulating influence of cyclic nucleotides upon lymphocyte-mediated cytotoxicity. *J. exp. Med.* **138**, 381.

STULTING R.D., BERKE G. & HIEMSTRA K. (1973) Evaluation of the effects of cytochalasin B on lymphocyte-mediated cytolysis. *Transplantation*, **16**, 684.

VISCHER T.L. (1972) Effect of hydrocortisone on the reactivity of thymus and spleen cells of mice to *in vitro* stimulation. *Immunology*, **23**, 777.

WERKELE H., LONAI P. & FELDMAN M. (1972) Fractionation of antigen-reactive cells on a cellular immuno-adsorbent: factors determining recognition of antigens by T lymphocytes. *Proc. nat. Acad. Sci. (Wash.)*, **69**, 1620.

WILSON L. (1970) Properties of colchicine binding protein from chick embryo brain. Interactions with vinca alkaloids and podophyllotoxin. *Biochemistry*, **9**, 4999.

WINCHURCH R.A., FOSCHI G.V. & WALTZ D.T. (1974) Inhibition of the lymphocyte-mediated cytotoxic reaction by anti-inflammatory drugs. *J. Reticuloend. Soc* **15**, 112.

WOLBERG G., HIEMSTRA K., BURGE J.J. & SINGLER R.C. (1973) Reversible inhibition of lymphocyte-mediated cytolysis by dimethyl sulfoxide (DMSO). *J. Immunol.* **111**, 1435.

ROSENAU W. & MOON H.D. (1966) Studies on the mechanism of the cytolytic effect of sensitized lymphocytes. *J. Immunol.* **96**, 80.