Immunology, 1970, **18**, 517.

Studies of Allograft Immunity in Mice

II. MECHANISM OF TARGET CELL INACTIVATION *In Vitro* BY SENSITIZED LYMPHOCYTES

J. MAUEL,* H. RUDOLF, B. CHAPUIS AND K. T. BRUNNER

Swiss Institute for Experimental Cancer Research, Lausanne, Switzerland

(*Received* 18*th July* 1969)

Summary. The mechanisms of the *in vitro* interaction of sensitized lymphocytes and allogeneic target cells has been studied in a tumour allograft system in inbred mice. The cytotoxic effect of sensitized lymphocytes is shown to require the presence of Ca^{++} and Mg^{++}. Pretreatment of the lymphocytes with trypsin led to inhibition of cytotoxicity, followed by spontaneous reversal after 1–3 hours incubation. Reactivation was found to be blocked by an inhibitor of protein synthesis (cycloheximide). Cortisone was not found to inhibit the lytic interaction significantly; an occasional effect is thought to be due to toxicity of cortisone for lymphocytes as revealed by dye exclusion test. Inhibition of DNA-synthesis with FUdR (an inhibitor of the enzyme thymidine synthetase) did not reduce the lytic activity of sensitized lymphocytes.

Isologous anti-target cell sera induced in various strains of inbred mice were found to be ineffective in blocking the cellular immune reaction *in vitro* when directed against a minor part of the antigenic complex, but strongly inhibitory when reactive against a major part or the whole complex. Similarly, target cells lacking several of the sensitizing *H*-2 antigens were not lysed. An isologous anti-lymphocytic serum induced in the graft donor strain and directed against the recipient strain (lymphocyte donor) did not inhibit the cytotoxic reaction. In a heterologous system on the other hand, the lytic effect of guinea-pig lymphocytes sensitized against mouse target cells was effectively blocked by an anti-lymphocytic serum induced in mice of the graft donor strain by injection of recipient (guinea-pig) spleen cells.

INTRODUCTION

Specifically sensitized lymphocytes from recipients of histoincompatible grafts may destroy antigenic target cells *in vitro*. The cytotoxic effect does not require the addition of complement or serum to the system. Reports from this and other laboratories indicate that target cell populations are lysed within relatively short time periods (1–3 hours) when adequate experimental systems are used (Friedman, 1964; Brunner, Mauel, Rudolf and Chapuis, 1970), that the rate of destruction is depressed by inhibitors of RNA and protein synthesis (Wilson, 1965; Brunner, Mauel, Cerottini and Chapuis, 1968), and that both 7S and 19S isoantibodies prepared in the graft recipient strongly inhibit the cytotoxic reaction (Brunner *et al.*, 1968).

* Present address: The Wistar Institute, Philadelphia, Pennsylvania 19104, U.S.A.

So far, the mechanisms of the cytotoxic activity of immune lymphocytes have remained essentially speculative. The requirement for close contact between the sensitized cells and their targets (Rosenau, 1963; Wilson, 1965a, b), and the inhibitory action of specific isoantibodies (attributed to competition with the lymphocytes for the same antigenic determinants of the target cells), suggest the presence of complementary antibody-like structures at the lymphocyte surface. However, attempts to block these hypothetical configurations with antigenic extracts have so far been unsuccessful (Brunner *et al.*, 1968).

This report presents a series of *in vitro* experiments in which the assay system described in earlier communications (Brunner *et al.*, 1968, 1970) has been applied to the investigation of several aspects of cellular immunity. It will be shown that sensitized lymphocytes are reversibly inactivated by trypsin, and that the cytotoxic process requires the presence of Ca^{++} and Mg^{++} in ionic form. Cortisone was not found to inhibit the reaction significantly, and DNA synthesis is not required for the lymphocytes to express their cytotoxic potential. In addition, the specificity of target cell lysis was analysed by evaluating the effect of sensitized lymphocytes on cells from inbred strains of mice other than the donor strain, and by testing the inhibitory effect of isoantisera reacting with only part of the *H*-2 specificities against which the lymphocytes were sensitized. The results of these experiments suggest that for effective lysis, the majority or all of the sensitizing *H*-2 antigens have to be either present or available on the target cell surface.

Finally, it was found that in the system described, isologous antilymphocyte sera induced in the donor strain (by injection of lymphocytes from the recipient strain) do not inhibit the cytotoxic effect, suggesting that target cell lysis involves more that an interaction with structurally different configurations at the lymphocyte surface.

MATERIALS AND METHODS

Animals and immunizations

To obtain sensitized allogeneic lymphocytes or isoantisera, inbred C57BL, C3H or A/Sn mice were immunized with DBA/2 P-815-X2 mastocytoma cells following methods described in the first part of this communication (Brunner *et al.*, 1970). To obtain sensitized heterologous lymphocytes, outbred guinea-pigs were immunized by one intraperitoneal injection of 15×10^7 mastocytoma cells, and spleens were harvested 11 days later. Suspensions of lymphocytes from immunized and control animals were prepared following methods described earlier (Brunner *et al.*, 1968). To obtain heterologous antisera: (a) DBA/2 mice were given three intraperitoneal injections of a total of 15×10^7 guinea-pig spleen cells; (b) rabbits were given 10^8 C57BL spleen cells half intraperitoneally, and half intramuscularly in Freund's complete adjuvant, followed by four intraperitoneal injections of a total of 4×10^8 of the same cells; and (c) BD IV or Lewis rats were given 6×10^7 DBA/2 mastocytoma cells intraperitoneally. All sera were inactivated by heating at 56° for 30 minutes. The DBA/2 anti-guinea-pig sera, and the normal DBA/2 control serum used in the same experiment were absorbed with an equal volume of packed guinea-pig erythrocytes at 4° for 30 minutes.

In vitro *assay of cellular immunity*

The cytotoxic effect of sensitized lymphocytes on ^{51}Cr-labelled target cells was measured following methods described in detail in the first part of this communication (Brunner *et al.*, 1970).

Antibody titrations

Agglutination titres of isologous and heterologous antisera against DBA/2 mastocytoma cells, DBA/2 and C57BL lymph node cells, or guinea-pig lymph node cells were determined by mixing, in 8×80 mm tubes, equal volumes (0·1 ml) of a suspension of 2×10^6/ml DBA/2 mastocytoma cells (or 5×10^6/ml lymph node cells) in Eagle's medium without serum, and serial serum dilutions in buffered saline. After incubation at 37° for 2 hours, the tubes were shaken and agglutination read directly in the tubes under the low power of an inverted microscope.

Incorporation of radioactive precursor into spleen cells

To 1-ml volumes of 10^7/ml spleen cells, 0·1 ml of a solution of tritiated cytidine ([^{3}H]CdR) (5 μCi/0·16 μg/ml) was added. After incubation for 2 hours at 37°, the cells were collected by centrifugation, washed with PBS, resuspended in 0·2 ml RNAse solution (50 μg/ml RNAse, 0·01 M EDTA in Tris-buffered saline), and frozen/thawed three times. After incubation for 2 hours at 37° 0·2 ml of 10 per cent TCA was added, and the preparations kept overnight at 4°. The precipitates were then placed on Whatman GF/A glass fibre filters, washed with 5 per cent TCA followed by 96 per cent ethyl alcohol, dried and the radioactivity measured in a liquid scintillation counter.

RESULTS

EFFECT OF TRYPSIN ON THE CYTOTOXIC ACTIVITY OF SENSITIZED LYMPHOCYTES

In order to explain the specificity of cellular immunity, several investigators have postulated a recognition step mediated by antibody-like configurations on the lymphocyte surface. To study whether proteolytic agents would modify surface structures so as to impair the lytic activity of immune lymphocytes, spleen cells of C57BL mice immunized with allogeneic (DBA/2) mastocytoma cells and spleen cells of normal control mice were incubated in a 0·2 per cent trypsin solution (crystallized trypsin, Difco, in phosphate

TABLE 1

EFFECT OF TRYPSIN PRETREATMENT OF SENSITIZED LYMPHOCYTES ON THE LYTIC INTERACTION *in vitro* WITH ^{51}Cr-LABELLED ALLOGENEIC TARGET CELLS

Experiment No.	Duration of incubation (hours)	Per cent specific label release by target cells incubated with lymphocytes pretreated with:		Per cent reduction by trypsin of specific ^{51}Cr release
		Trypsin	Buffer alone	
1	3	14	38	63
	6	55	73	25
	9	71	79	10
2	3	24	40	40
	6	67	71	6
	9	85	81	0
3	3	18	47	62
	6	55	84	35
	9	76	85	10

Sensitized (or normal) lymphocytes incubated for 30 minutes at 37° in a 0·2 per cent trypsin solution or in buffered saline. Cells washed and mixed with ^{51}Cr-labelled target cells at a ratio of 100 : 1. Per cent specific label release determined after 3, 6 and 9 hours incubation.

buffered saline, pH 7·4) for 30 minutes at 37°, and then washed in Eagle's medium supplemented with 10 per cent calf serum to stop the reaction. Normal and sensitized spleen cells incubated in buffer alone served as controls. Dye exclusion tests showed that trypsin was not toxic for the lymphocytes. The various cell suspensions were then incubated with ^{51}Cr-labelled DBA/2 mastocytoma target cells at the usual ratio of 100 viable lymphocytes/target cell. Cytotoxicity was assayed following techniques described in detail in the first part of this communication (Brunner *et al.*, 1970).

In three experiments in which the lytic effect of trypsinized lymphocytes was measured after 3 hours incubation, the enzyme treatment was found to reduce specific ^{51}Cr release by 40–63 per cent when compared to buffer treatment (Table 1). However, the observed inhibition of the cytotoxic activity was greatly reduced when the reaction time was extended to 6 hours (6–35 per cent). An analysis of the kinetics of ^{51}Cr release revealed that the rate of lysis had changed, and that the trypsin-treated cells had regained the activity of the controls in the second period of incubation (3–6 hours). When a highly sensitized lymphocytic population was used in a fourth experiment, the observed delay was shortened and full reactivity was regained after 1 hour (Fig. 1).

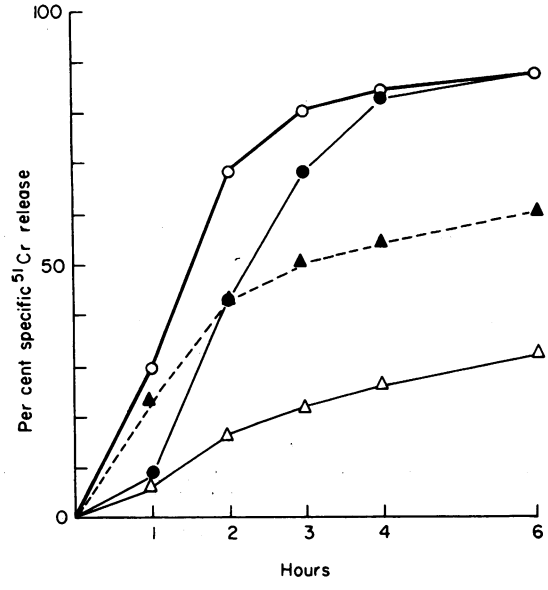

FIG. 1. Effect of trypsin treatment of sensitized lymphocytes (and/or of blocking of protein synthesis) on the lytic interaction with ^{51}Cr-labelled allogeneic target cells. Sensitized (or normal) lymphocytes incubated for 30 minutes at 37° in a 0·2 per cent trypsin solution or in buffered saline. Cells washed and mixed with ^{51}Cr-labelled target cells at a ratio of 100 : 1 in the presence or absence of 100 μg/ml cycloheximide. Per cent specific release of label measured in time. ○, Control; ●, trypsin; ▲, cycloheximide; △, trypsin + cycloheximide.

The transitory reduction of the cytotoxic activity after trypsin treatment may be explained by the inactivation of specific recognition sites and/or other protein components contributing to the reaction. On the other hand, the recovery of the lytic capacity could be attributed to the reappearance of the missing proteins at the lymphocyte surface, either by diffusion from an intracellular pool and/or by *de novo* synthesis. If the latter hypothesis were true, inhibitors of protein synthesis should extend the effect of trypsin.

The possible maintenance of a depressed state of cytotoxic activity was, therefore, studied. Reaction mixtures of trypsin-treated sensitized lymphocytes and target cells were incubated in the presence of 10 μg/ml of cycloheximide, an inhibitor of protein synthesis. The same preparations without drug, and untreated sensitized spleen cells with the inhibitor, were included in the test. As shown in Fig. 1, the addition of cycloheximide to trypsin-treated lymphocytes resulted in an indefinite prolongation of the blocking of the lytic activity induced by the enzyme. In three experiments, the cytotoxicity of trypsin-treated lymphocytes incubated with target cells in the presence of the drug for 6 hours was reduced by 62, 69 and 87 per cent when compared to untreated controls in a drug-free medium, while the percentages of inhibition due to the antimetabolite alone, or trypsin alone, were 46, 36 and 56 per cent, and 5, 1 and 34 per cent respectively.

INHIBITION OF THE CELLULAR IMMUNE REACTION WITH EDTA

The possible role of metal ions in the development of the cytotoxic reaction was investigated by means of the chelating agent EDTA. Preliminary experiments showed that this chemical is effective in preventing the cytotoxic effect *in vitro* of sensitized lymphocytes. In order to determine the lowest effective concentration of the compound, various amounts of its trisodium salt were added to reaction mixtures of sensitized or normal lymphocytes (1×10^7/ml) and labelled target cells (1×10^5/ml) suspended in Eagle's medium supplemented with 10 per cent calf serum. Reaction mixtures containing sensitized or normal lymphocytes without added EDTA served as controls to test for possible toxicity of the agent for target cells.

The amount of label released was determined in each preparation after 6 hours incubation. As can be seen in Table 2, complete inhibition of the cytotoxic reaction was obtained with 0·005 M EDTA, at which concentration this compound did not present any toxic effect on the target cells during the incubation times involved, as judged from the comparison with the amounts of ^{51}Cr released in the control suspensions.

TABLE 2

EFFECT OF EDTA ON THE LYTIC INTERACTION OF SENSITIZED LYMPHOCYTES AND ^{51}Cr-LABELLED ALLOGENEIC TARGET CELLS

Experiment No.	Final concentration of EDTA in reaction mixture	Per cent ^{51}Cr release by target cells incubated with immune lymphocytes during 6 hours	
		In presence of EDTA	In absence of EDTA
	–	–	61
1	0·0001 M	64,5	–
	0·001 M	64,5	–
	0·01 M	0	–
	–	–	66
2	0·001 M	77	–
	0·005 M	1	–
	0·01 M	0	–
3	–	–	74
	0·001 M	74	–
	0·005 M	1	–
	0·01 M	0	–

Sensitized (or normal) lymphocytes and target cells at a ratio of 100 : 1 incubated in the presence or absence of EDTA. Percent specific label release determined after 6 hours incubation.

While the presence of certain metal ions seems to be necessary for the immune reaction, it does not necessarily follow that these ions are directly involved in the cytotoxic process. Chelating agents such as EDTA, by altering cellular metabolism may affect the structural integrity of the lymphocytes and thereby indirectly inhibit their cytotoxic activity.

Experiments were therefore performed to test for the possible reversibility of the EDTA-induced inhibition. Sensitized lymphocytes (2×10^7/ml in Eagle's medium containing serum) were exposed for 3 hours at 37° to EDTA concentrations ranging from 0·001 to 0·01 M, washed by centrifugation and mixed in the same medium with ^{51}Cr-labelled mastocytoma cells at a ratio of 100 lymphocytes/target cell.

Specific ^{51}Cr release was determined after 3 and 6 hours by comparison with the amount of label recovered from suspensions containing target cells and similarly treated normal lymphocytes. In other experiments, reversion of the blocking effect was tested by adding Ca^{++} and Mg^{++} ions to culture fluids without removal of the chelating agent: reaction mixtures of sensitized lymphocytes and target cells were incubated for 3 hours in the presence of EDTA; excess Ca^{++} and Mg^{++} ions were then added to the suspensions and the reaction allowed to proceed at 37°. The amount of ^{51}Cr liberated was then recorded after different periods of time.

TABLE 3

EFFECT OF EDTA PRETREATMENT OF SENSITIZED LYMPHOCYTES FOLLOWED BY WASHING OR ADDITION OF EXCESS Ca^{++} AND Mg^{++} ON THE LYTIC INTERACTION WITH ^{51}Cr-LABELLED ALLOGENEIC TARGET CELLS

Experiment No.	Final concentration of EDTA during 3 hours pre-incubation of lymphocytes	Reversal of the effect of EDTA: washing or addition of Ca^{++}/Mg^{++}	Per cent ^{51}Cr released by target cells incubated in presence of EDTA-pre-treated lymphocytes during	
			3 hours	6 hours
1	0·001 M	Washing	51	80
	0·005 M	Washing	49	82
	0·01 M	Washing	42·5	80
2	0·001 M	Add $CaCl_2$ 0·0015 mM/ml + $MgCl_2$ 0·0015 mM/ml	75	71
	0·005 M	Add $CaCl_2$ 0·0075 mM/ml + $MgCl_2$ 0·0075 mM/ml	40	72
	0·01 M	Add $CaCl_2$ 0·015 mM/ml + $MgCl_2$ 0·015 mM/ml	36	70
	No EDTA	–	56	73
2	0·005 M	Add $CaCl_2$ 0·0075 M/ml + $MgCl_2$ 0·0075 M/ml	37	–
	No EDTA	–	44	–

Sensitized (or normal) lymphocytes incubated for 3 hours in the presence of various concentrations of EDTA. Cells washed by centrifugation, or excess Ca^{++} and Mg^{++} added. Lymphocytes mixed with labelled target cells at a ratio of 100 : 1. Per cent specific label release measured after 3 and 6 hours' incubation.

As shown in Table 3, EDTA-treated sensitized spleen cells recovered full reactivity upon washing or addition of excess Ca^{++} and Mg^{++} ions. The lytic effect of treated preparations was slightly lower than that of untreated controls after 3 hours, but the same toxicity levels were reached in both suspensions when the incubation time was extended to 6 hours.

In an effort to analyze more closely the inhibitory action of the chelating agent, EDTA was added to reaction mixtures of sensitized lymphocytes and target cells at a time intermediate between onset and completion of the cytotoxic reaction. In the same experiments, the ability of either Ca^{++} or Mg^{++} to reverse EDTA-induced inhibition was compared to the effect of a mixture of both ions.

The results of a typical experiment are presented in Fig. 2. When EDTA was added 45 minutes after the beginning of the incubation, the cytotoxic activity of the preparations reached 73 per cent of the control level after 6 hours, while in two other experiments, addition of EDTA 60 and 120 minutes after the onset of the cytotoxic reaction failed to induce any significant reduction of the lytic activity.

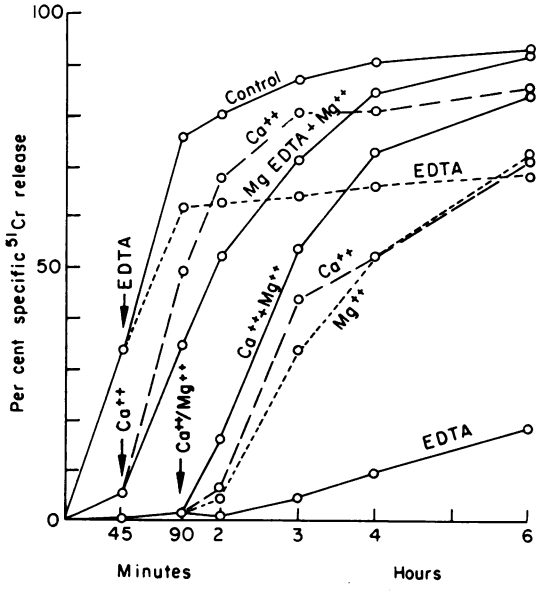

FIG. 2. Inhibition by EDTA of the lytic interaction of sensitized lymphocytes with ^{51}Cr-labelled allogeneic target cells; test for reversal of effect upon addition of Ca^{++}, Mg^{++} or Ca^{++} plus Mg^{++}. Sensitized lymphocytes and ^{51}Cr-labelled target cells mixed at a ratio of 100 : 1 and incubated in the presence or absence of 0·005 M EDTA, or incubated in the presence of 0·01 M EDTA + 0·015 mM/ml Mg^{++}. After 45 minutes incubation, to part of the tubes which received no additions, 1·005 mM/ml EDTA was added. After 45 minutes' incubation, to part of the tubes containing Mg EDTA + Mg^{++}, Ca^{++} (0·015 mM/ml) was added. After 90 minutes incubation, to part of the tubes containing EDTA, either Ca^{++} or Mg^{++} (0·015 mM/ml) or both (0·0075 mM/ml) each was added. Specific release of label measured at the time intervals indicated.

As shown in Fig. 2, the addition of either Ca^{++} or Mg^{++} ions to EDTA-treated reaction mixtures restored the cellular toxicity to a large extent. Although the reversal induced by either ion was of the same magnitude, the addition of Ca^{++} or Mg^{++} singly was found in four experiments to be somewhat less effective than a mixture of both. In addition, it can be seen in Fig. 2 that the selective complexing of Ca^{++} by the chelating agent Mg-EDTA delayed the cytotoxic reaction, even in the presence of excess Mg^{++}. However, the terminal level of lysis was not affected.

INABILITY OF CORTISONE TREATMENT TO PREVENT THE CYTOTOXIC ACTIVITY *in vitro*

Addition of cortisone to reaction mixtures of sensitized lymphocytes and target cells has been reported to inhibit cytotoxic effects on the latter, as measured by microscopic examination of the cultures after 48 hours' incubation (Rosenau and Moon, 1962). In order to study this phenomenon more closely, two cortisone derivatives (Ultracorten-H and Hydrocortisone, CIBA, Basle, Switzerland) were added at various concentrations to

mixtures of sensitized lymphocytes and labelled target cells: Ultracorten-H dissolved readily in culture fluids and was used more extensively.

In five preliminary experiments, specific release of ^{51}Cr in reaction mixtures of immune spleen cells and target cells at a ratio of 100 : 1 was measured in the presence or absence of various concentrations of cortisone, and release of label was determined after 6 hours incubation. Control suspensions of normal lymphocytes and target cells were included with and without cortisone to test for toxicity of the drug for target cells. No such effect was observed.

In two of these experiments, various concentrations of hydrocortisone were tested, and no inhibition of target cell lysis was observed up to 10 μg (100 μg)/ml i.e. to 82 per cent (87 per cent) lysis in the absence of drug corresponded 81 per cent (90 per cent) in the presence of 10 μg (100 μg) hydrocortisone/ml.

In the following three experiments, Ultracorten-H at concentrations of 0·25–25 μg/ml was found slightly to inhibit specific release of ^{51}Cr (10 per cent inhibition at all concentrations tested). In one of the experiments, final concentrations of 50, 150, and 250 μg/ml were included, which showed a somewhat stronger effect which was again independent of the dose (20 per cent inhibition for all three concentrations).

The possible toxicity of cortisone for lymphocytes was then tested by dye exclusion tests on cells incubated for 6–24 hours with various concentrations of the drug. After 6 hours a slight toxicity could be observed at hydrocortisone concentrations of 1·0 and 10·0 μg/ml, which reached significant levels after 24 hours. In two experiments, lymphocyte survival after 24 hours was 6 per cent (4 per cent) at 10 μg/ml, and 6 per cent (6 per cent) at 1 μg/ml, while control populations showed survival of 43 per cent (42 per cent) in the absence of drug. In additional experiments, the same concentrations of hydrocortisone (1·0 and 10·0 μg/ml) were found to be non-toxic for ^{51}Cr-labelled mastocytoma cells, i.e.

TABLE 4

EFFECT OF HYDROCORTISONE AND OF ULTRACORTEN-H ON: (a) *in-vitro* LYMPHOCYTE SURVIVAL, AND (b) ON THE LYTIC INTERACTION OF SENSITIZED LYMPHOCYTES AND ^{51}Cr-LABELLED ALLOGENEIC TARGET CELLS

Drug added at final concentration (μg/ml)	Per cent lymphocyte survival (dye exclusion test) after incubation for:				Per cent specific ^{51}Cr release by target cells in the presence of sensitized lymphocytes at the ratio of:					
					100 lymphocytes/target cell after incubation for:			30 lymphocytes/target cell after incubation for:		
	2 hr	4 hr	6 hr	24 hr	2 hr	4 hr	6 hr	2 hr	4 hr	6 hr
Experiment 1: Hydrocortisone										
100·0	78	67	46	< 1	50	73	79	30	58	72
10·0	80	73	58	< 1	56	76	80	32	58	71
1·0	89	76	61	< 1	59	76	83	32	59	73
–	86	86	76	27	61	80	82	37	67	77
Experiment 2: Ultra-corten-H										
100·0	85	77	72	10	76	88	90	54	76	80
10·0	96	84	63	12	78	83	89	57	80	82
1·0	93	80	71	12	78	87	89	62	79	81
–	87	80	79	52	78	86	89	66	82	88

(a) Lymphocyte suspensions (10^7/ml) incubated in the presence or absence of cortisone. Trypan blue exclusion tests performed after various time intervals (microscopic counts of 2 × 250 cells) in duplicate assay tubes. (b) Sensitized (or normal) lymphocytes and ^{51}Cr-labelled target cells mixed at ratios of 100/1 and 30/1 and incubated in the presence (or absence) of cortisone. Specific release of label measured after the time interval indicated.

these cells released the same amount of label in 24 hours when incubated in the presence or absence of the drug.

Table 4 shows the results of two combined experiments, in which the time course of target cell lysis by sensitized spleen cells in the presence or absence of either hydrocortisone or Ultracorten-H was followed at ratios of 100 and 30 lymphocytes/target cell, and toxicity of the drug for lymphocytes was measured in parallel. Target cell lysis by sensitized lymphocytes was again only very slightly affected. In the first experiment, ^{51}Cr release appeared to be somewhat retarded by all concentrations of hydrocortisone tested (1, 10 and 100 μg/ml), both at ratios of lymphocytes to target cells of 100 : 1 and 30 : 1. In the second experiment, in which lysis in the absence of drug was more rapid than in the first one, Ultracorten-H showed a similar effect only at the ratio of 30 : 1. Toxicity of the drugs for the spleen cells assayed by dye exclusion test, again reached significant levels after 24 hours incubation at all concentrations of hydrocortisone and Ultracorten-H tested.

The results suggest that in the system described, cortisone may slightly inhibit the cellular immune reaction *in vitro* by its toxicity for lymphocytes.

EFFECT OF FUdR ON THE CYTOTOXIC ACTIVITY OF IMMUNE LYMPHOCYTES

Experiments were carried out to determine whether blocking of DNA synthesis would affect the cytotoxic activity of immune lymphocytes. Spleen cells from immunized and control animals were incubated in the presence of various concentrations of FUdR (an inhibitor of the enzyme thymidine synthetase) for 1 hour at 37° in Eagle's medium supplemented with 10 per cent dialysed calf serum. Without further washing, the lymphocytes were then mixed with labelled target cells at the usual ratio of 100 : 1, and the percentage of specific lysis in 2 hours at 37° was determined. The incorporation of [^{3}H]deoxycytidine (3·5–5 μc/ml) by treated and untreated spleen cell suspensions was recorded in parallel experiments as a measure of the inhibition of DNA synthesis (see 'Materials and methods').

As shown in the results summarized in Table 5, the cytotoxic activity of sensitized lymphocytes was not inhibited by FUdR at any of the concentrations tested. Although in

TABLE 5

EFFECT OF FUdR ON (a) THE LYTIC INTERACTION OF SENSITIZED LYMPHOCYTES AND ^{51}Cr-LABELLED ALLOGENEIC TARGET CELLS, AND (b) ON ^{3}H-CYTIDINE ([^{3}H]CdR) INCORPORATION BY SPLEEN CELLS

Experiment No.	FUdR added	Per cent specific ^{51}Cr release in 2 hours by target cells incubated with sensitized spleen cells	[^{3}H]CdR incorporation in 2 hours by spleen cells	
			counts/min	Per cent inhibition
1	2×10^{-4}M	65·0	2471	56
	6×10^{-5}M	62·7	3430	39
	2×10^{-5}M	65·0	5245	6
		64·4	5611	–
2	2×10^{-4}M	71·4	731	54
	6×10^{-5}M	68·4	837	48
	2×10^{-5}M	70·3	1014	37
		67·7	1608	–

(a) Sensitized lymphocytes and ^{51}Cr-labelled target cells at a ratio of 100 : 1 incubated in the presence or absence of FUdR. Per cent specific release of label measured after 2 hours incubation, based on total ^{51}Cr incorporation in target cells = 100 per cent. (b) Lymphocyte suspensions (10^7/ml) incubated for 2 hours in the presence of 5 μCi/0·16 μg/ml [^{3}H]CdR.

Incorporation of [^{3}H]CdR (counts/min) measured in a liquid scintillation counter.

these experiments cytidine incorporation was only partially blocked (a residual incorporation of 40–50 per cent of the control values was always observed with the drug at a concentration of 2×10^{-4}M), the fact that target cell lysis was not inhibited suggests that DNA synthesis is not necessary to sustain the cytotoxic activity of immune lymphocytes.

INHIBITION OF THE CYTOTOXIC REACTION BY ISOANTISERA

Earlier experiments had shown that the cytotoxic properties of immune (C57BL antiDBA/2) lymphocytes was inhibited when anti-DBA/2 antibodies prepared in C57BL graft recipients were added to the reacting cells (Brunner, Mauel and Schindler, 1967; Brunner *et al.*, 1968). These results have been explained on the basis of a mechanism of competition between antibodies and lymphocytes for identical sites on the

TABLE 6

INHIBITORY EFFECT OF ANTI-TARGET CELL ISOANTISERA ON THE LYTIC INTERACTION OF SENSITIZED LYMPHOCYTES AND ^{51}Cr-LABELLED ALLOGENEIC TARGET CELLS

Per cent specific ^{51}Cr release in 6 hours by DBA/2 target cells in the presence of:						
Sensitized C3H→DBA/2 lymphoid cells			Sensitized C57BL→DBA/2 lymphoid cells			Sensitized A/Sn→DBA/2 lymphoid cells
And C3H→DBA/2 serum 1	Experiment No. 1	2	And C57→DBA/2 serum 3	Experiment No. 1	2	And A/Sn→DBA/2 serum 2	Experiment 2
10%	34	21	10%	–	32	10%	22
3%	37	31	3%	–	47	3%	30
1%	45	40	1%	–	61	1%	48
0	80	70	0	–	81	0	80
10% normal C3H serum	77	75	10% normal C57 serum	–	83	10% normal A/Sn serum	78
And A/Sn→DBA/2 serum 2	Experiment No. 1	2	And A/Sn→DBA/2 serum 2	Experiment No. 1	2	And C57BL→DBA/2 serum 3	Experiment 2
10%	84	75	10%		80	10%	26
3%	77	72	3%	–	80	3%	44
1%	72	70	1%	–	77	1%	56
0	80	70	0	–	81	0	80
10% normal A/Sn serum	82	71	10% normal A/Sn	–	77	10% normal C57BL serum	74
And C57BL→DBA/2 serum 3	Experiment No. 1	2	And C3H→DBA/2 serum 1	Experiment No. 1	2	And C3H→DBA/2 serum 1	Experiment 2
10%	29	30	10%	0	28	10%	22
3%	34	42	3%	19	45	3%	34
1%	65	57	1%	71	61	1%	49
0	80	70	0	94	81	0	80
10% normal C57BL serum	78	77	10% normal C3H serum	93	82	10% normal C3H serum	76

Sera 1, 2 and 3, agglutination titre against DBA/2 mastocytoma cells: 1280.

Sensitized lymphocytes and target cells at a ratio of 100 : 1 incubated for 6 hours in the presence of various concentrations of isoantiserum, of normal allogeneic serum, and without serum added, and percent specific label release measured.

target cell surface. The specificity of this protection was supported by the demonstration that antibodies induced against target cells in a third strain of mice (A/Sn) were unable to prevent the lytic effect. We now present additional data extending these previous findings.

Immune lymphocytes and anti-target cell antibodies were prepared by intraperitoneal injections of DBA/2 mastocytoma cells into C57BL, A/Sn, and C3H mice. Table 7 indicates the various *H*-2 antigens against which the lymphocytes may become sensitized, and with which the antisera may react (Snell, Hoecker and Amos, 1964). Sera prepared in all three strains reached titres of 1 : 1280 at the end of the immunization course, as measured by agglutination tests performed on the mastocytoma cells. Similarly, after one immunizing injection, sensitized lymphocyte populations of all three sources had comparable cytotoxic activities when tested against DBA/2 target cells.

Sensitized spleen cells from each source were then assayed in the cytotoxicity test against labelled mastocytoma cells, in the presence or absence of various dilutions of the three antisera. As expected, each antiserum inhibited the cytotoxic effect of immune lymphocytes of syngeneic origin (Tables 6 and 7), but the properties of the three sera were found to differ when assayed on allogeneic lymphocytes. A/Sn anti-DBA/2 serum at any concentration tested was unable to inhibit the destruction of target cells confronted with either C57BL or C3H lymphocytes. In contrast, C57BL and C3H antisera were almost as effective in blocking the activity of allogeneic lymphocytes as the antiserum prepared in the strain syngeneic with the lymphocytes tested.

TABLE 7

SUMMARY OF THE INHIBITORY EFFECT OF ANTI-TARGET CELL ISOANTISERA SHOWN IN TABLE 6, IN RELATION TO THE *H*-2 ALLOANTIGENS AGAINST WHICH THE LYMPHOCYTES MAY BE SENSITIZED AND THE *H*-2-ALLOANTIGENS WHICH MAY BE COVERED BY THE ISOANTISERA

Lymphocytes tested on target cells	Sensitized lymphocytes may react with DBA/2 target cell *H*-2-antigens	Inhibition of target cell lysis by isoantiserum	Effect of anti-serum	Antibody may react with *H*-2-antigens	Sites covered by antibody/sites attacked by lymphocytes
C3H→DBA/2	4, 6, 10, 13, 14, 27, 28, 29, 31	C3H→DBA/2	+	4, 6, 10, 13, 14, 27, 28, 29, 31	9/9
		A→DBA/2	−	31	1/9
		C57BL→DBA/2	+	4, 10, 13, 31	4/9
C57BL→DBA/2	3, 4, 8, 10, 13, 31	C57BL→DBA/2	+	3, 4, 8, 10, 13, 31	6/6
		A→DBA/2	−	31	1/6
		C3H→DBA/2	+	4, 10, 13, 31	4/6
A→DBA/2	31	A→DBA/2	+	31	1/1
		C57BL→DBA/2	+	31	1/1
		C3H→DBA/2	+	31	1/1

Alloantigenic specificities based on table published by Snell *et al.* (1964).

These results show that the A/Sn anti-DBA/2 serum which covers only a minor part of the *H*-2 specificities against which the lymphocytes may react (one of six or one of nine) does not block the cytotoxic effect of either C3H anti-DBA/2 or C57 anti-DBA/2 lymphocytes, while the antisera which cover a major part of the target cell antigens (four of nine for C57BL anti-DBA/2 and four of six for C3H anti-DBA/2 sera) protect against attack by C3H anti-DBA/2 and C57BL anti-DBA/2 lymphocytes respectively. Table 7 summarizes the results in relation to the *H*-2 specificities which are presumably involved.

If partial covering of antigenic sites by isoantibody is effective in blocking lymphocyte toxicity, then availability on a target cell of only part of the *H*-2 antigen complex against which the lymphocytes are sensitized may similarly preclude effective interaction (Brondz, 1968). This can readily be verified by measuring the lytic effect of the sensitized lymphocytes on target cells of the various strains in which the partially reactive isoantisera had been induced. If for example, C57BL anti-DBA/2 lymphocytes, which may theoretically react with six *H*-2 specificities of the DBA/2 donor strain (3, 4, 8, 10, 13 and 31) are tested against C3H target cells, only two of the six *H*-2 antigens (3 and 8) are available for attack (while the C3H anti-DBA/2 serum was reactive against the other four antigens). It may then be predicted that the C57BL anti-DBA/2 lymphocytes do not recognize the antigens 3 and 8 of the C3H target cells.

Such an experiment, testing C3H anti-DBA/2, C57BL anti-DBA/2 and A anti-DBA/2 lymphocytes on ^{51}Cr-labelled mouse embryo fibroblast (MEF) target cells of all three strains is presented in Table 8. The results demonstrate that the sensitized lymphocytes were indeed not cytotoxic for target cells which carry only part (two of six or five of nine) of the *H*-2 specificities against which they are presumably sensitized and thus confirm similar observations of Brondz (1968). In addition, the results agree with the predictions based on the inhibitory effect of the corresponding isoantisera. In contrast to this apparent lack of antigenic recognition by (or lack of sensitization of) the recipient lymphocytes, serological assays showed that erythrocytes of the C3H strain were agglutinated by a C57BL anti-DBA/2 serum (titre 1 : 512) and C57BL erythrocytes were agglutinated by a C3H anti-DBA/2 serum (titre 1 : 1024), although to titres about fifteen-fold lower than the ones obtained with the respective donor red blood cells (titres 1 : 8000 and 1 : 16,000, respectively).

EFFECT OF HOMOLOGOUS AND HETEROLOGOUS ANTI-LYMPHOCYTE SERA (ALS)

Homologous ALS

It has been reported that unsensitized, immunologically competent cells may become cytotoxic for allogeneic or semisyngeneic target cells upon addition of PHA. It was further demonstrated that the PHA-mediated cytotoxicity could be partially or totally abolished by pretreatment of the lymphocytes with homologous antisera raised in the target cell donor strain (Möller, 1967). These findings had lent support to the hypothesis that the lytic effect of PHA-treated lymphocytes was due to confrontation of target cells with foreign structures on the lymphocyte surface (Möller and Möller, 1965).

Experiments were carried out to investigate the possible protective effect of homologous anti-lymphocyte serum (ALS) on the cytotoxicity *in vitro* of immune lymphocytes. Anti-C57BL sera were raised in DBA/2 mice by repeated intraperitoneal injections of C57BL spleen cells until the HA titres reached 1 : 1024 to 1 : 2048. These homologous ALS were added to mixtures of sensitized or normal C57BL spleen cells and labelled DBA/2 target cells, and specific release of ^{51}Cr determined after 6 hours incubation at 37°. The effect of anti-target cell sera at similar titres was measured in parallel experiments to compare the protective effect in both systems.

Upon addition of anti-C57BL antiserum to the suspensions, a strong agglutination of the spleen cells could be observed. However, no inhibition of the cytotoxic activity could be detected in five experiments in which the effect of various anti-lymphocyte sera was tested. Indeed, the amount of ^{51}Cr released by target cells in the presence of immune

TABLE 8

LYTIC EFFECT OF SENSITIZED LYMPHOCYTES FROM C3H, C57BL AND A MICE IMMUNIZED WITH DBA/2 MASTOCYTOMA CELLS ON ^{51}Cr-LABELLED MOUSE EMBRYO FIBROBLASTS OF ALL FOUR STRAINS

Sensitized lymphocytes	Target cells (MEF)	Per cent specific ^{51}Cr release by target cells in:			No. of *H*-2 sites available sensitized lymphocytes
		2 hr	4 hr	6 hr	
C3H→DBA/2	DBA/2	33	52	59	9/9
	C57BL	–	–	2	5/9
	A	15	30	38	8/9
	C3H	0	0	0	0/9
C57BL→DBA/2	DBA/2	36	53	58	6/6
	A	21	35	42	5/6
	C3H	2	2	2	2/6
	C57BL	–	–	0	0/6
A→DBA/2	DBA/2	11	23	32	1/1
	C57BL	–	–	0	0/1
	C3H	0	0	0	0/1

Sensitized lymphocytes and target cells at a ratio of 100 : 1 incubated for 2,4 and 6 hours. Percent specific label release measured, based on total ^{51}Cr incorporation in target cells = 100 per cent. Number of *H*-2 sites available for attack by lymphocytes indicated, in relation to the *H*-2 specificities against which the lymphocytes may be sensitized.

lymphocytes and anti-lymphocyte isoantiserum was increased by 8–38 per cent when compared to the release in reaction mixtures without added antiserum. In the same experiments, anti-target cell isoantisera were added to reaction mixtures at HA titres similar to those of the anti-lymphocyte isoantisera. These sera induced partial or total inhibition of the cellular immune reaction *in vitro*, as described before (Möller, 1965; Brunner *et al.*, 1967, 1968). The results of three representative experiments are shown in Table 9.

TABLE 9

COMPARISON OF THE INHIBITORY EFFECT OF ANTI-TARGET CELL AND OF ANTI-LYMPHOCYTE ISOANTISERA ON THE LYTIC INTERACTION OF SENSITIZED LYMPHOCYTE AND ^{51}Cr-LABELLED TARGET CELLS

Experiment No.	Haemagglutination titre of anti-lymphocyte serum	Per cent specific ^{51}Cr-release, in 6 hours, by DBA 2 target cells incubated in the presence of sensitized C57BL spleen cells and 10 per cent			Haemagglutination titre of anti-target cell serum
		Anti-lymphocyte serum (DBA2→C57BL)	No anti-serum	Anti-target cell serum (C57BL→DBA2)	
1	2048	89	82	–	–
2	2048	84	79	61	410
3	1024	85	73	26	8000
			73	51	800

Anti-target cell serum raised in C57BL recipient strain by immunization with DBA/2 donor target cells. Anti-lymphocyte serum raised in DBA/2 donor strain by immunization with C57BL recipient spleen cells. Percent specific ^{51}Cr release measured after 6 hours incubation in the presence or absence of antiserum.

Thus anti-lymphocyte sera directed against the histocompatibility antigens of the spleen cells which are absent from the target cells did not protect the latter against the cytotoxic activity of the lymphocytes. These results suggest that, in the lytic process mediated by sensitized lymphocytes. the close contact of target cells with structurally different moieties on the surface of the attacking cells is not the factor responsible for their destruction.

Heterologous ALS

The *in vitro* system described above was thought to represent a useful tool with which to evaluate not only the effect of isologous, but also of heterologous anti-lymphocyte sera on the cytotoxicity of sensitized lymphocytes. In preliminary experiments, the respective activities of two sera were tested in parallel: (a) of anti-mouse serum raised in rabbits by several injections of C57BL mouse spleen cells (see 'Materials and methods'), and (b) of a rat anti-target cell serum obtained by repeated intraperitoneal administration of DBA/2 mastocytoma cells. Both sera effectively inhibited target cell lysis by sensitized lymphocytes, but the results of agglutination tests indicated that both sera could react with either type of cell present in the reaction mixtures. Since earlier experiments had shown that coating of target cells with specific isoantibodies entirely blocked the cytotoxic reaction, the protective effect observed with the 'anti-lymphocyte' serum could not be attributed with certainty to an action at the lymphocyte level.

A new experimental system was thus designed, in which the heterologous ALS would react exclusively with the attacking lymphocytes. Guinea-pigs were sensitized against DBA/2 mastocytoma target cells (see 'Materials and methods') and anti-guinea-pig lymphocyte serum was induced in DBA/2 mice by repeated intraperitoneal injection of guinea-pig spleen cells.

Reaction mixtures containing normal or sensitized guinea-pig lymphocytes were incubated in the presence of various dilutions of inactivated mouse anti-guinea-pig lymphocyte serum. Controls included suspensions with and without normal mouse serum. An inhibitory effect of normal DBA/2 mouse serum on the cytotoxic reaction, observed in early experiments, could be suppressed by absorption of the serum with guinea-pig red blood cells: thus absorption of normal sera and of antisera immediately before use was adopted as a standard procedure. The agglutination titres of the antisera, tested with guinea-pig lymphocytes, were also determined only after absorption with guinea-pig red cells.

TABLE 10

EFFECT OF HETEROLOGOUS ANTI-LYMPHOCYTE SERUM RAISED IN DONOR (DBA/2) MICE BY IMMUNIZATION WITH RECIPIENT (GUINEA-PIG) SPLEEN CELLS ON THE LYTIC INTERACTION OF SENSITIZED GUINEA-PIG LYMPHOCYTES WITH ^{51}Cr-LABELLED HETEROLOGOUS (DBA/2) MOUSE TARGET CELLS

	Per cent specific ^{51}Cr release in 9 hours by DBA/2 target cells in the presence of sensitized guinea-pig lymphocytes and DBA/2→guinea-pig serum*				
Antiserum added	10%	3%	1%	–	10% normal DBA/2 serum
Experiment 1	3	6	36	59	65
Experiment 2	2	32	79	77	78

Sensitized lymphocytes from guinea-pigs injected with DBA/2 mastocytoma cells and ^{51}Cr-labelled mastocytoma target cells at a ratio of 100 : 1 incubated in the presence of various concentrations of the anti-lymphocyte serum, of normal DBA/2 serum and in the absence of added serum. Sera absorbed with guinea-pig erythrocytes. Per cent specific release of label measured after 6 hours incubation.

* Agglutination titre for guinea-pig lymph node cells = 640.

In five experiments, the cytotoxic effect of sensitized guinea-pig lymphocytes was reduced by 93–100 per cent when anti-guinea-pig lymphocyte serum was added to the reaction mixtures at a final concentration of 10 per cent. This protection still reached 59–96 per cent at a 3 per cent antiserum concentration, and 0–69 per cent at a 1 per cent concentration. The results of two representative experiments are shown in Table 10.

Trypan blue exclusion tests indicated that the viability of the lymphocytes was not affected by the antisera. Thus, the inhibitory effect of the anti-lymphocyte sera, which in the system described could only react with the attacking lymphocytes, was not based on a toxic effect leading to the death of the sensitized cells.

DISCUSSION

Trypsin treatment of sensitized lymphocytes has been shown to result in a reversible inhibition of their cytotoxic activity. This phenomenon can best be explained by the unbinding or degradation of a protein moiety of the lymphocyte membrane necessary for the cytotoxic process. It would be tempting to recognize in this material the antibody-like configuration of the lymphocyte membrane postulated in the first article (Brunner *et al.*, 1970): however, the exact nature and role of the trypsin-sensitive substance has yet to be established.

Upon elimination of the enzyme, the lymphocytes recovered full activity in a short time, as demonstrated by the fact that, following the initial period of inhibited lysis, the destruction of target cells returned to a rate similar to the one found in suspensions containing untreated lymphocytes.

The complete reversion of the inhibitory effect of trypsin can be attributed to the reappearance of the missing moiety on the lymphocyte surface, either by *de novo* synthesis and/or by diffusion from a pre-existing intracellular pool. In a previous report, we have shown that the cytotoxic activity of sensitized lymphocytes is, at least partially, dependent on protein synthesis (Brunner *et al.*, 1968). When reaction mixtures of trypsin-treated lymphocytes and target cells were incubated in the continuous presence of cycloheximide, target cell lysis was not abolished, but it continued at a greatly reduced rate throughout the incubation period. This reduction of activity by cycloheximide (as compared to the effect of trypsin alone) is probably due to impaired synthetic processes unable to reconstitute the missing material in sufficient concentration. Conversely, the residual cytotoxicity observed in such preparations may be due to an incomplete degradation of the active substance, and/or its reappearance on the cell surface by transport from a preformed pool.

Similar studies by Brondz and Bartova (1966), led these authors to conclude that trypsinization of sensitized lymphocytes did not alter their cytotoxic potential as long as their viability was not affected. This discrepancy from the results presented in this report can be adequately explained on the basis of differing experimental systems. The blocking effect of the enzyme can only be detected during a short period following treatment: in the assay system described by these authors, the cytotoxic effect was assayed after 24–48 hours of incubation of the reaction mixtures, a period long enough for full reversal of the effect of the enzyme.

A parallel can be drawn between the action of trypsin in *in vitro* models of cellular transplantation immunity and of delayed hypersensitivity. As demonstrated by David, Lawrence and Thomas (1964), trypsin treatment of sensitized lymphocytes inhibits the blocking effect of antigen on macrophage migration. As in the present report, this effect was also shown to be temporary, since trypsin-treated lymphocytes recovered full reactivity after incubation for 24 hours in enzyme-free medium.

Complement mediated lysis of antibody-coated target cells is known to require the presence of Ca^{++} and Mg^{++}. It can thus be blocked by the addition of chelating agents

which remove these ions from the solution. In an analysis *in vitro* of hypersensitivity phenomena, Lichtenstein and Osler (1964) established that EDTA also prevents the release of histamine from peripheral blood leukocytes of allergic individuals challenged with the specific antigen. The lysis of target cells by sensitized lymphocytes, as described in this report, is still another immunological event which can be prevented by Ca^{++} and Mg^{++} depletion.

Almost complete reversal was observed when either Ca^{++} or Mg^{++} was added to the system, and selective removal of Ca^{++} with MgEDTA had only a slightly inhibitory effect. This demonstrates that lysis by sensitized lymphocytes may occur at low concentrations of Ca^{++}, as has been described for the complement system when human serum was used as a complement source (Marney and Des Prez, 1969).

In view of the results obtained with inhibitors of RNA and protein synthesis, which showed that the blocking of anabolic activities in sensitized lymphocytes results only in a partial reduction of their cytotoxic potential (Brunner *et al.*, 1968), it is unlikely that the very strong inhibitory action of EDTA was mediated by an effect on the synthetic pathways of the cells. On the other hand, while both the cytotoxic activity of lymphocytes and histamine release from blood leukocytes are inhibited by EDTA, neither requires the presence of exogeneous complement. The participation of complement cannot be ruled out entirely, however. It has in fact been shown that lymphocytes may lyse adequately sensitized red blood cells by supplying factors of the complement system (Perlmann, Perlmann, Manni and Müller-Eberhard, 1969).

In the hypersensitivity phenomenon described above, the effect of the EDTA was not dependent on the time of addition, since the release of histamine could be terminated upon chelation of the divalent cations at any time after the onset of the reaction. In contrast, EDTA could not block the cytotoxic activity of lymphocytes once the lytic reaction was initiated. The point of action of the chelating agent would thus be expected at an early stage in the lytic process. It is conceivable for instance, that it interferes with the specific attachment of the lymphocytes to the target cells.

Using the assay system described in this report, cortisone was not found to inhibit the cytotoxic activity of sensitized lymphocytes to any important degree. This result is in disagreement with previous findings by Rosenau and Moon (1962), who were able to show a strongly inhibitory effect of the drug in a somewhat different experimental model measuring the cytotoxic effect of lymphocytes by microscopic counts of target cell nuclei after 24 hours incubation. However, while these authors did not find any toxic effect of the drug on lymphocytes, the data presented indicate that such an effect occurs in our system. This toxicity is thought to be responsible for the slight inhibition of target cell lysis observed at the higher concentrations of Ultracorten-H.

The protective action of specific anti-target cell antibodies against the cytotoxic action of immune lymphocytes is well established (Möller, 1965; Brunner *et al.*, 1967, 1968) and is adequately explained by a mechanism of competition with the lymphocytes for the same antigenic determinants at the target cell surface. The data presented in this report indicate that this effect can be achieved not only by antibodies of syngeneic origin with regard to the lymphocytes, but also, in certain cases, by antibodies raised in allogeneic animals. Thus, C57BL anti-DBA/2 and C3H anti-DBA/2 antibodies both inhibit the activity of C57BL, C3H or A/Sn lymphocytes, while A/Sn anti-DBA/2 antibodies could only protect the target cells against A/Sn lymphocytes.

Assuming that: (a) in the combination tested, non *H*-2 differences play only a minor

role in the graft rejection process, and (b) that the DBA/2 target (and immunizing) cells do not bear tumour antigens (which seems likely, the first article, Brunner *et al.*, 1970), the results obtained in this study can best be analysed in the light of the known differences at the *H*-2 loci of the four strains (Table 7). It must be emphasized, however, that the specificities reported in Table 7 have been drawn mostly from agglutination experiments (i.e. involving antigens on red blood cells): the status of the various nucleated cells is not known, and the validity of applying these data to cellular immunology is still questionable. A further important restriction is that many specificities listed in Table 7 do not appear as the result of single immunizations between two strains, but are attributed to particular strains because of cross-reactive patterns detected by use of unrelated isoantisera (Stimpfling and Richardson, 1965).

According to the present state of knowledge, the sole difference between strains A/Sn and DBA/2 is at the single allele 31 (E^d). Thus, immunization against the product of a single *H*-2 specificity can be sufficient to induce a very strong immunity, as demonstrated by the cytotoxic potential of lymphocytes (Table 6) and antiserum. That this cytotoxicity is blocked by treatment of the target cells with any of the three anti-DBA/2 sera can be attributed to the fact that, very likely, each serum contains antibodies specific for the product of this allele (Table 7). Conversely, A/Sn anti-DBA/2 serum is unable to protect DBA/2 target cells against C57BL or C3H lymphocytes because, according to Table 7, it lacks antibodies against several DBA/2 specificities, which have determined a state of immunity in C57BL and C3H mice.

This situation becomes more complex, however, when the reciprocal protection induced by C3H and C57BL antisera against C57BL and C3H lymphocytes is interpreted on the same basis. Either serum offers inhibitory activity in spite of the fact that neither one can react with all of the specificities theoretically susceptible to attack by the challenging lymphocytes. Moreover, the level of protection afforded by both anti-target cell sera against allogeneic lymphocytes equals the protection obtained by use of the syngeneic combination. Supporting evidence in favour of the effectiveness of partial covering of the antigenic complex is provided by the observation (Table 8) that target cells of the strain in which the partially reactive antisera were induced were not lysed by the lymphocytes sensitized against the larger number of *H*-2 specificities of the DBA/2 donor strain.

Three hypotheses can account for these results: (a) the immunization procedures did not result in a sensitization of the lymphocytes against all the specificities which differentiate the *H*-2 loci of two given strains; (b) the specificity of the lymphocytes is directed against the whole antigen complex rather than against individual antigens, as suggested by Brondz (1968); or (c) all *H*-2 specificities are spatially grouped close together, so that the fixation of antibodies on an important part of the *H*-2 complex could interfere, by steric hindrance, with the necessary mechanisms by which lymphocytes exert their lytic activity. This explanation agrees with findings recently reported by Cresswell and Sanderson (1968), which indicate that the fixation of antibodies on part of the *H*-2 molecule does inhibit the subsequent capacity of this molecule to fix antibodies directed toward other *H*-2 specificities.

On the other hand, it does not explain the lack of lytic activity of lymphocytes presumably sensitized against six or nine *H*-2 antigens when confronted with target cells of third party strains carrying only two of the six or five of the nine specificities. Antibodies reacting with erythrocytes of these third party strains were formed by the recipients, demonstrating the presence and immunogenicity of the corresponding antigens. The

results thus suggest either lack of adequate sensitization of the lymphocytes, or lack of antigenic recognition when several specificities are either absent or covered by antibody.

The phenomenon of allogeneic inhibition has been described as a non-specific cytotoxicity mediated by lymphocytes and other cell types aggregated by phytohaemagglutinin (PHA) to unrelated target cells. Since it had been observed that parental target cells were destroyed by F_1 hybrid lymphocytes, the lytic effect was thought to involve non-immunological mechanisms: it was thus suggested that structural differences on the surface of the reacting cells were responsible for the phenomenon (Möller and Möller, 1965). This hypothesis was further strengthened by the demonstration that isoantibodies induced in the graft donor against the lymphoid cells could prevent their PHA-mediated cytotoxicity (Möller, 1967). In our own experiments, DBA/2 anti-C57BL isoantibodies failed to inhibit or even reduce the lytic action of C57BL lymphocytes on DBA/2 target cells.

The recent findings by Perlmann, Perlmann and Holm (1968), that the lytic activity of PHA stimulated lymphocytes is not restricted to allogeneic cells, but, upon suitable conditions, is effective against syngeneic cells as well, also suggests that the cytotoxic activity of lymphocytes is probably not due to the mere contact of target cells with structurally different configurations of the lymphocyte surface.

The mechanisms by which anti-lymphocyte serum delays or suppresses graft rejection are not yet fully understood. Although ALS administration has been shown to induce a profound depletion of circulating lymphocytes, lymphopenia alone cannot account for some particular manifestations of ALS treatment (Levey and Medawar, 1966). For instance, it has been suggested that antiserum coats lymphocytes in such a way as to render their combining sites inaccessible to further stimuli, or unable to operate their normal functions in the course of a lytic interaction with target cells.

The validity of this hypothesis was first examined by incubating mixtures of sensitized lymphocytes and target cells in the presence of various anti-lymphocyte sera, in complement-free fluids. An anti-mouse lymphocyte serum raised in rabbits by repeated immunization with mouse spleen cells proved to be a strong inhibitor of the cytotoxic reaction: however, as this serum was found to bind to target cells as well, this approach failed to supply conclusive evidence with regard to the effect of lymphocyte coating. Another experimental system was thus devised in which mixtures of guinea-pig anti-mouse lymphocytes were incubated with DBA/2 mouse target cells in culture fluids containing mouse anti-guinea-pig serum. The results presented in Table 10 show that in a heterologous system where antibodies reacted with lymphocytes only, an almost complete inhibition of the lytic activity could be obtained.

Addendum. Since this paper was submitted, it was found that the cytotoxicity of sensitized guinea-pig lymphocytes for DBA/2 mastocytoma cells is strongly inhibited by rabbit antisera directed against guinea-pig immunoglobulin G (IgG), while in the allogeneic system, no effect of rabbit antiserum to mouse IgG, IgA, IgM or whole serum was noted (Chapuis and Brunner, unpublished observation). This suggests a possible rôle of sensitizing antibody formed in the heterologous system by the immune lymphocytes incubated with target cells.

ACKNOWLEDGMENT

This work was supported by the Swiss National Foundation for Scientific Research.

REFERENCES

BRONDZ, B. D. (1968). 'Complex specificity of immune lymphocytes in allogeneic cell cultures.' *Folia Biol. (Praha)*, **14**, 115.

BRONDZ, B. D. and BARTOVA, L. M. (1966). 'A study of the influence of certain factors on the activity of lymphocytes in allogeneic macrophage cultures.' *Folia Biol. (Praha)*, **12**, 431.

BRUNNER, K. T., MAUEL, J., CEROTTINI, J. C. and 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. and CHAPUIS, B. (1970). 'Studies on allograft immunity in mice. I. Induction, development and *in vitro* assay of cellular immunity.' *Immunology*, **18**, 499.

BRUNNER, K. T., MAUEL, J. and SCHINDLER, R. (1967). 'Inhibitory effect of isoantibody on *in-vivo* sensitization and on the *in-vitro* cytotoxic action of immune lymphocytes.' *Nature (Lond.)*, **213**, 1246.

CRESSWELL, P. and SANDERSON, A. R. (1968). 'Spatial arrangement of H-2 specificities: evidence from antibody absorption and kinetic studies.' *Transplantation*, **6**, 996.

DAVID, J. R., LAWRENCE, H. S. and THOMAS, L. (1964). 'The *in-vitro* desensitization of sensitive cells by trypsin.' *J. exp. Med.*, **120**, 1189.

FRIEDMAN, H. (1964). 'Inhibition of antibody plaque formation by sensitized lymphoid cells: rapid indicator of transplantation immunity.' *Science*, **145**, 607.

LEVEY, R. H. and MEDAWAR, P. B. (1966). 'Some experiments on the action of antilymphoid antisera.' *Ann. N.Y. Acad. Sci.*, **129**, 164.

LICHTENSTEIN, L. M. and OSLER, A. G. (1964). 'Studies on the mechanisms of hypersensitivity phenomena. IX. Histamine release from human leucocytes by ragweed pollen antigen.' *J. exp. Med.*, **120**, 507.

MARNEY, S. R. and DES PREZ, R. M. (1969). 'Comparison of the effects of divalent ion chelating agents on complement-mediated hemolysis in rabbit and human serum.' *J. Immunol*, **103**, 1044.

MÖLLER, E. (1965). 'Antagonistic effect of humoral isoantibodies on the *in-vitro* cytotoxicity of immune lymphoid cells.' *J. exp. Med.*, **122**, 11.

MÖLLER, E. (1967). 'Cytotoxicity by nonimmune allogeneic lymphoid cells.' *J. exp. Med.*, **126**, 395.

MÖLLER, G. and MÖLLER, E. (1965). 'The cytotoxic effect of antigenic and/or structural incompatibility *in-vitro*.' *Isoantigens and Cell Interactions* (Ed. by J. Palm), pp. 65–70. The Wistar Institute Press, Philadelphia.

PERLMANN, P., PERLMANN, H., MANNI, G. and MÜLLER-EBERHARD, H. (1969). 'Cytotoxic effects of leucocytes triggered by target cell bound complement.' *Science*, **163**, 937.

PERLMANN, P., PERLMANN, H. and HOLM, G. (1968). 'Cytotoxic action of stimulated lymphocytes on allogeneic and autologous erythrocytes.' *Science*, **160**, 306.

ROSENAU, W. (1963). 'Interaction of lymphoid cells with target cells in tissue culture.' *Cell-Bound Antibodies* (Ed. by B. Amos and H. Koprowski), pp. 75–83. Wistar Inst. Press, Philadelphia, Pennsylvania.

ROSENAU, W. and MOON, H. D. (1962). 'The inhibitory effect of hydrocortisone on lysis of homologous cells by lymphocytes *in-vitro*.' *J. Immunol.*, **89**, 422.

SNELL, G. D., HOECKER, G. and AMOS, B. D. (1964). 'A revised nomenclature for the histocompatibility-2 locus of the mouse.' *Transplantation*, **2**, 277.

STIMPFLING, G. H. and RICHARDSON, A. (1965). 'Recombination within the histocompatibility—2 locus of the mouse.' *Genetics*, **51**, 831.

WILSON, D. B. (1965a). 'Quantitative studies on the behaviour of sensitized lymphocytes *in vitro*. II. Inhibitory influence of the immune suppressor, Imuran, on the destructive reaction of sensitized lymphoid cells against homologous target cells.' *J. exp. Med.*, **122**, 167.

WILSON, D. B. (1965b). 'Quantitative studies on the behaviour of sensitized lymphocytes *in-vitro*. I. Relationship of the degree of destruction of homologous target cells to the number of lymphocytes and to the time of contact in culture and consideration of the effects of isoimmune serum.' *J. exp. Med.*, **122**, 143.