Immunology, 1968, **14**, 181.

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

K. T. Brunner, J. Mauel, J.-C. Cerottini and B. Chapuis

Swiss Institute for Experimental Cancer Research, Lausanne, and Institute of Biochemistry, University of Lausanne, Switzerland

(*Received* 15*th May* 1967)

Summary. The *in vitro* cytotoxic effect of spleen cells of mice immunized by tumour allografts was studied by measuring target cell inactivation as a function of release of radioactive label (^{51}Cr) or loss of cloning efficiency. When sensitized lymphoid cells were incubated with target cells at a ratio of 100:1, up to 90 per cent of the incorporated label was released within 6–9 hours, while the number of clone-forming cells was reduced by up to 99 per cent in the same time period. Isoantiserum from the graft recipients, as well as its 19S and 7S fractions, protected target cells against the toxic effect of the spleen cells, but a lipoprotein antigen isolated from the tumour cells failed to inhibit the cytotoxic reaction. Target cell lysis as measured by specific release of ^{51}Cr was partially inhibited by actinomycin-D and by cycloheximide at concentrations which effectively blocked DNA-dependent RNA and protein synthesis.

INTRODUCTION

The demonstration that lymphoid cells from immune animals attack and destroy target cells *in vitro* provides simplified systems with which to study cellular transplantation immunity and tumour immunity. Assay systems measuring and analysing the cytotoxic effect of immune lympoid cells have been described by several authors. In these studies, the fate of the target cells was followed by microscopic observation and cell counts (for references see: Wilson, 1965a), antibody plaque formation (Friedman, 1964), cloning assay (Brunner, Mauel and Schindler, 1966), and release of radioactive label (Vainio, Koskimies, Perlmann, Perlmann and Klein, 1964). The results so far obtained demonstrate that close contact with living, sensitized lymphoid cells is necessary (Rosenau, 1963) and leads to target cell inactivation within 6–12 hours (Friedman, 1964; Brunner *et al.*, 1966). Conventional cytotoxic antibody and complement appear not to be involved in the reaction: in fact, the addition of isoantibody may even protect the target cells from attack (Möller, 1965; Brunner, Mauel and Schindler, 1967b).

Several reports have stressed the importance of the metabolic integrity of lymphocytes participating in cellular immune reactions. High doses of X-irradiation (15,000 r) inactivated sensitized lymphoid cells (Rosenau and Moon, 1966); likewise, Imuran was found to inhibit their cytotoxic effect *in vitro* (Wilson, 1965b), and results suggesting a similar effect of puromycin were reported by Calabresi, Brunner and Mauel (1967). Delayed hypersensitivity *in vitro* as measured by inhibition of macrophage migration was

suppressed by inhibition of protein synthesis (David, 1965), but analogous studies with lymphoid cells sensitized by histocompatibility antigens to our knowledge have not been reported.

To study some of these questions, we have developed a simple and highly sensitive *in vitro* assay system of cellular immunity which is based on allogeneic target cell lysis by sensitized lymphoid cells as measured by release of radioactive chromium (^{51}Cr) from pre-labelled target cells. In the presence of immune lymphoid cells, target cells were found to release up to 90 per cent of the label within 6–9 hours. These data confirm earlier experiments demonstrating loss of cloning efficiency within similar incubation periods. In addition, the application of the system has allowed the quantitative evaluation of the effect of selected inhibitors of protein and of nucleic acid synthesis and the effect of isoantibody and microsomal lipoprotein antigen on the reaction.

It will be shown that specific isoantibody directed against target cells effectively inhibits their lysis by sensitized spleen cells, but that the antigen extracts so far tested have no inhibitory activity. Inhibition of protein synthesis and of DNA-dependent RNA synthesis depressed, but did not abolish the cytotoxic effect of sensitized lymphoid cells.

MATERIALS AND METHODS

Immunization by tumour allograft

Following the methods described in detail in an earlier communication (Brunner *et al.*, 1966) female C57BL mice were sensitized by one intraperitoneal injection of 3×10^7 DBA/2 mastocytoma P-815-X2 cells (Dunn and Potter, 1957) in ascitic form. Spleen cells of the recipient mice were harvested 9–12 days later.

Spleen cell suspensions

Spleens of recipient and untreated control C57BL mice were removed aseptically, cut in a few fragments, and homogenized by hand in a glass Ten-Broek grinder with modified Eagle's medium (four-fold amounts of amino acids and vitamins) without serum added. The cell suspension obtained was immediately centrifuged for 5 minutes at 1500 rev/min, the sediment re-suspended in the same medium and left standing for 1 hour at 4°. The sediment which had formed was then discarded, the cells in the supernatant fluid washed twice and finally re-suspended in Eagle's medium containing 10 per cent inactivated calf serum. The number of viable cells was determined by haemocytometer counts using the Trypan-blue exclusion test, and the suspension adjusted to 2×10^7 viable cells/ml. The number of viable cells varied between 80 and 95 per cent of the total cell count. Spleen cells from immunized mice will be described as 'immune' or 'sensitized' spleen or lymphoid cells.

Target cells

The DBA/2 mastocytoma cells described above were cultured *in vitro* in modified Eagle's medium supplemented with 10 per cent calf serum. The cells growing in suspension were fed every 2 or 3 days by dilution.

Labelling of target cells

Mastocytoma cells grown in suspension cultures were harvested, suspended in Tris-buffered saline and labelled with ^{51}Cr (Sanderson, 1965). Volumes of 0·4 ml of the cell

suspension containing 2×10^6 cells/ml were incubated with 40 μc of radioactive sodium chromate ([^{51}Cr]sodium chromate, specific activity 100–400 μc/μg of chromium, Radiochemical Centre, Amersham, England) for 30 minutes at 37°. The cell suspension was then centrifuged for 5 minutes at 1000 rev/min, and re-suspended in modified Eagle's medium containing 10 per cent inactivated calf serum. After five washings of the pellet in this medium, the cell suspension was adjusted to 2×10^5 viable cells/ml as determined by Trypan-blue exclusion test and haemocytometer count.

Cytotoxicity tests with sensitized lymphoid cells

The medium used in all cytotoxicity tests consisted of modified Eagle's medium supplemented with 10 per cent inactivated calf serum.

(a) *^{51}Cr release.* Spleen cell suspensions containing 2×10^7 cells/ml were mixed with equal volumes of pre-labelled target cell suspensions containing 2×10^5 cells/ml. One millilitre of the reaction mixtures thus obtained was placed in each of several Leighton tubes, and the cells incubated at 37° for the specified time period. For the assay of ^{51}Cr released into the supernatant fluid, 1 ml of Eagle's medium was added to each of the Leighton tubes followed by centrifugation at 1500 rev/min for 5 minutes. Volumes of 1·5 ml of the supernatant fluid of each tube were then carefully removed for measurement of radioactivity in a scintillation counter. The result was multiplied by 4/3 to correct for the volume left with the sediments.

The cytotoxic effect of the sensitized lymphoid cells was expressed by the difference between release in samples containing sensitized lymphoid cells and samples containing normal allogeneic lymphoid cells.

For each sample, the percentage of ^{51}Cr release was calculated, based on the total amount of label incorporated in the target cells, i.e.:

$$\frac{\text{^{51}Cr release in the presence of immune lymphoid cells} - \text{^{51}Cr release in the presence of normal lymphoid cells}}{\text{Total ^{51}Cr incorporated} - \text{^{51}Cr release in the presence of normal lymphoid cells}} \times 100.$$

The term inhibition was used to describe the protective effect of antiserum and drugs on the cytotoxic reactions. Per cent inhibition was calculated on the basis of difference in label release between reaction mixtures containing sensitized lymphoid cells and antiserum (or drug) as compared to the same mixtures in the absence of serum (or drug). Inhibition was determined as per cent reduction of the cytotoxic effect taken as 100 per cent. Corrections were made for ^{51}Cr release due to toxic effects of serum (or drug) as determined in control reaction mixtures containing normal lymphocytes and serum (or drug) at the concentrations of the test.

No cytotoxic effect of normal allogeneic spleen cells was noted under the conditions of the test; in five experiments release ranged between 13 and 17 per cent after 6 hours incubation. On the other hand, in some of the experiments, a slight increase in ^{51}Cr release was noted in the presence of 10 per cent hyperimmune C57BL anti-DBA/2 serum (haemagglutination titre 1:8000–1:16,000), as well as in the presence of 10 μg/ml of cycloheximide, while 10 μg/ml of actinomycin showed no such effect.

(b) *Reduction of cloning efficiency.* Spleen cell suspensions containing 2×10^7 cells/ ml were mixed with equal volumes of target mastocytoma cell suspensions containing 2×10^5 cells/ml. One-millilitre volumes of these reaction mixtures were placed in Leighton tubes and after varying periods of incubation, the number of surviving target cells was

determined by cloning assay following the technique described in a previous communication (Brunner *et al.*, 1966).

Haemagglutination and cytotoxicity tests of isoantisera

Haemagglutination tests were carried out following the method described by Stimpfling (1961), but with agglutination evaluated under a low power microscope.

The cytotoxicity tests were made according to Sanderson (1964). Lysis of ^{51}Cr-labelled mastocytoma cells was evaluated by comparing the number of counts/min released into the supernatant fluid with the maximum release obtained with a positive control serum. Maximum release (= 100 per cent lysis) was found to correspond to 75–80 per cent of the total label incorporated into the tumour cells. The results were corrected for spontaneous release in the presence of normal serum and complement. *For cytotoxicity inhibition studies*, antigen dilutions were tested against a standard dose of cytotoxic antiserum showing 70–80 per cent lysis in the presence of control antigen or buffer. The inhibition titres correspond to the dilutions showing 50 per cent inhibition of lysis.

Incorporation of radioactive precursors into spleen cells

Tubes with 1×10^7/ml sensitized or normal spleen cells were prepared, to which either 5 μc/ml of [^{3}H]uridine (25 mc/mM, NEN, Boston, Massachussetts) or 0·5 μc of [^{14}C]leucine (25 mc/mM, NEN, Boston, Massachussetts) were added. The tubes were incubated, and samples removed at intervals. The cells were centrifuged and washed twice with Tris-buffered saline. The sediment was then re-suspended in 10 per cent trichloracetic acid (TCA), the precipitate washed once with the TCA, suspended in distilled water, and placed on filter paper discs. The dried discs were placed in scintillation counting fluid, and the radioactivity determined in a scintillation counter (Nuclear, Chicago).

Preparation of 19S and 7S isoantibody fractions

Isoimmune sera were prepared in C57BL mice by four to six intraperitoneal injections of 3×10^7 living DBA/2 mastocytoma ascites cells at about 1-week intervals. The sera were fractionated by gel filtration on Sephadex G-200 columns equilibrated with 0·02 M phosphate buffer in 0·3 M NaCl, pH 7·4. Elution was carried out with the same buffer at a flow rate of 22 ml/hr, the 19S and 7S regions pooled and concentrated by pressure dialysis against 0·01 M phosphate buffer in 0·15 M NaCl, pH 7·4, to a volume corresponding to the initial volume.

The 19S fractions submitted to reduction or reduction and alkylation did not show haemagglutinating or cytotoxic activity, while treatment with iodoacetamide or buffer only did not affect serological reactivity.

Reduction–alkylation. The fractions were diluted with 8 volumes of 0·01 M phosphate buffer in 0·15 M NaCl, pH 7·4, and 1 volume of 1·0 M 2-mercaptoethanol. After incubation for 60 minutes at 37°, 1 volume of 1·0 M iodoacetamide was added, and the samples placed in an ice bath for 30 minutes. The excess iodoacetamide was then removed by dialysis against a large volume of 0·01 M phosphate buffer in 0·15 M NaCl, pH 7·4, for 12 hours at 4°.

Preparation of lipoprotein antigen

Mastocytoma cells in ascitic form were harvested, washed twice with Eagle's medium

without serum, and re-suspended in 0·25 M buffered sucrose (0·25 M sucrose; 10^{-2} M $MgCl_2$; 10^{-2} M Tris, pH 7·4). The suspension was placed in nitrocellulose centrifuge tubes, gassed with nitrogen for 5 minutes, and placed in an ice bath for sonication with a Branson Model S-125 sonifier. Sonication cycles of 15 seconds each were repeated at 1-minute intervals until most of the cells were lysed. The method of Manson, Foschi and Palm (1963) was then followed to obtain the lipoprotein fraction.

RESULTS

In earlier studies it was found that one intraperitoneal injection of 3×10^7 allogeneic DBA/2 mastocytoma cells into C57BL recipient mice regularly induced a high level of cellular immunity within 8–9 days (Brunner, Mauel and Schindler, 1967a).

In many experiments, microscopic observation of reaction mixtures containing 100

TABLE 1

COMPARISON OF THE CYTOTOXIC EFFECT OF SENSITIZED C57BL LYMPHOID CELLS ON DBA/2 TARGET CELLS AS MEASURED BY CLONING ASSAY AND BY RELEASE OF RADIOACTIVE LABEL

Experiment No.	Per cent ^{51}Cr released by target cells when incubated with sensitized lymphoid cells for:			Per cent inhibition of cloning efficiency after incubation for:	
	3 hours	6 hours	9 hours	3 hours	6 hours
1	31	62	77		
2	40	77	91		
3	50	79	–		
4	50	90	–		
5	18	53	70		
6	20	48	65		
7	19	41	–	53	84
8	37	61	–	65	87
9	31	48	–	71	99
10	34	68	–	–	84
11	39	81	–	–	79
12	28	66	80	58	91

sensitized lymphoid cells per target cell revealed actual target cell lysis after 6 hours incubation. This finding suggested the use of ^{51}Cr release from labelled target cells as a direct quantitative measure of lysis.

CYTOTOXICITY ASSAYED BY ^{51}Cr RELEASE

In a number of experiments, the cytotoxic effect of sensitized lymphoid cells was determined by measuring the difference in amount of label released by target cells in the presence of sensitized and of normal allogeneic spleen cells. When target cells were incubated for 3, 6 and 9 hours in the presence of 100 sensitized cells per target cell, progressive release of label occurred; 41–90 per cent of the total amount incorporated was released in 6 hours, and 65–91 per cent was released in 9 hours. The effect had already reached levels up to 50 per cent within 3 hours incubation (Table 1).

No lytic effect of normal allogeneic lymphoid cells was noted in any of the experiments.

When spontaneous release of ^{51}Cr by pre-labelled target cells in the absence of lymphocytes was compared to release in the presence of normal spleen cells, identical amounts of ^{51}Cr were found in the supernatant fluid of the cultures, reaching in 24 hours 25 per cent of total label incorporated. In spite of this apparent lack of 'allogeneic inhibition' (Hellström and Hellström, 1965; Möller and Möller, 1965) by normal lymphoid cells, all experiments measuring cytotoxicity of sensitized spleen cells carried out in these studies are based on comparison with control reaction mixtures containing normal lymphoid cells.

COMPARISON OF CLONING ASSAY AND ^{51}Cr RELEASE

To compare directly the rates of lysis and loss of cloning efficiency, a number of parallel cytotoxic tests were carried out, adding the same suspension of sensitized lymphoid cells to pre-labelled and to unlabelled mastocytoma cells. After 3, 6 and 9 hours, ^{51}Cr release and numbers of clonable cells were determined, respectively. Both assay methods demonstrated progressive target cell inactivation reaching significant levels at 3 hours incubation. The results of six such experiments are summarized in Table 1. Maximum reduction of clonable cells reached 99 per cent in 6 hours, and maximum release of ^{51}Cr reached 90 per cent in the same time period. The rates of inactivation as measured by the two methods were roughly parallel, although in one experiment (9), very rapid decrease in cloning efficiency was matched by a somewhat slower rate of label release.

DOSE EFFECT OF SENSITIZED LYMPHOID CELLS

To analyse the dose effect of sensitized lymphoid cells, increasing numbers of immune spleen cells were mixed with decreasing numbers of normal spleen cells. The final mixture in each case contained 2×10^7 viable lymphoid cells/ml. To each of these suspensions, an equal volume of a suspension of pre-labelled target cells containing 2×10^5 cells/ml was added. Release of radioactive label was determined after 3, 6 and 9 hours incubation, and it was compared to the amount released in the presence of 100 per cent normal spleen cells after each time interval. The results of one of these experiments are shown in Fig. 1. In the presence of 100 per cent sensitized lymphoid cells, specific release progressed linearly for 6 hours, reaching 62 per cent; the rate was then reduced to reach only 77 per cent in 9 hours. In the presence of 30 per cent and of 10 per cent sensitized spleen cells, the release was progressive up to 9 hours, reaching 47 and 16 per cent, respectively.

INHIBITORY EFFECT OF SPECIFIC ISOANTIBODY

Earlier experiments showed that specific isoantiserum produced by C57BL recipients of DBA/2 mastocytoma allografts effectively inhibits the cytotoxic action of the immunized animal's spleen cells against DBA/2 mastocytoma target cells *in vitro* (Brunner *et al.*, 1967b). The protective effect of the isoantibody is presumably due to competition with sensitized lymphoid cells for the same antigenic determinants at the target cell surface. However, antibody reacting with adjacent sites might also inhibit the reaction by a mechanism of steric hindrance.

Experiments were carried out to evaluate the cell protective effect of isoantiserum as measured by inhibition of ^{51}Cr release. Increasing amounts of inactivated hyperimmune

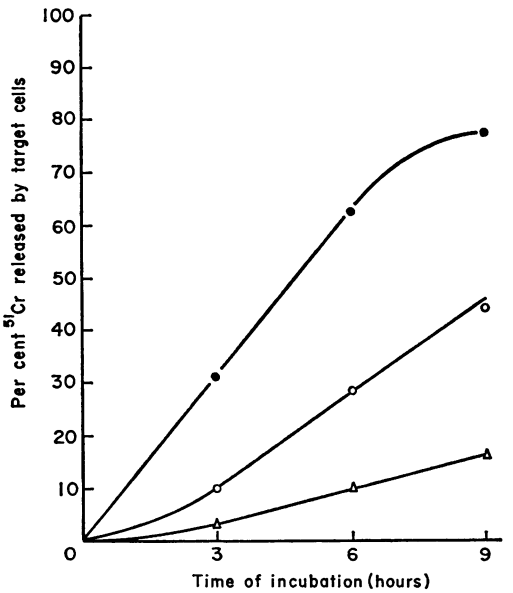

FIG. 1. Allogeneic target cell lysis by immune lymphoid cells. Mastocytoma cells at a concentration of 1×10^7/ml were incubated for 3, 6 and 9 hours with constant numbers (1×10^7) of lymphocytes containing 10, 30 or 100 per cent immune and 90, 70 or 0 per cent normal cells. Lysis was determined by measuring specific release of label by the target cells. ●, 100 per cent sensitive lymphocytes; ○ 30 per cent; △, 10 per cent.

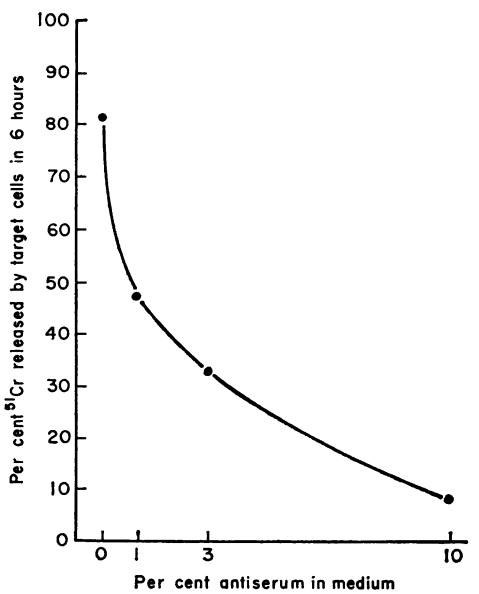

FIG. 2. Inhibitory effect of isoantiserum on target cell lysis by sensitized lymphoid cells. Increasing amounts of anti-mastocytoma isoantiserum were added to reaction mixtures containing 1×10^7 sensitized or normal lymphoid cells and 1×10^5 ^{51}Cr-labelled target cells. Lysis was determined after 6 hours incubation by measuring specific release of label by the mastocytoma cells.

C57BL anti-DBA/2 mastocytoma serum (HA-titre 16,000) were added to reaction mixtures containing either sensitized or normal spleen cells at multiples of 100 lymphoid cells per target cell. The reaction mixtures were incubated for 6 hours and the radioactivity released from the target cells measured for each cell suspension.

In two experiments, ^{51}Cr release caused by the cytotoxic action of sensitized spleen cells was almost completely inhibited by the addition of 10 per cent immune serum, i.e. 10, 3 and 1 per cent isoantiserum added to the reaction mixtures resulted in 90 and 91, 59 and 64, and 42 and 51 per cent inhibition in the two experiments. Detailed results of one of these experiments are shown in Fig. 2.

In most of the experiments, a slight toxicity of the antiserum (or its 19S and 7S fractions) as expressed by increased ^{51}Cr release in the control tubes containing normal lymphoid cells and target cells was noted. In eight experiments, spontaneous release of label by target cells incubated in the presence of normal spleen cells and 10 per cent inactivated isoantiserum was increased by 1–10 per cent (in a ninth experiment, 17 per cent) when compared to spontaneous release from the same cell suspension without added isoantiserum. This toxicity was corrected for in the determination of specific release.

Experiments were then performed to test the specificity of inhibition of lymphoid cell–target cell interaction by C57BL anti-DBA/2 isoantiserum, and if possible to exclude an effect of steric hindrance. Isoantisera were prepared by immunization of A/Sn mice with repeated injections of living DBA/2 mastocytoma cells. The A/Sn mice have been shown to lack only one H-2 antigen reported in DBA/2 mice, while the C57BL mice lack several H-2 antigens carried by DBA/2 mice (Amos, 1962). A/Sn anti-DBA/2 isoantibody was, therefore, thought to occupy antigenic sites at the target cell surface which differed to a large extent from the ones occupied by the C57BL anti-DBA/2 serum, and thus to serve as a control antibody in the inhibition studies. The A/Sn anti-DBA/2 isoantisera obtained showed no haemagglutinating activity with DBA/2 erythrocytes, but reached cytotoxic titres against DBA/2 mastocytoma cells of 2000–4000, i.e. similar to the titres of the C57BL anti-DBA/2 hyperimmune sera used in the inhibition studies described above. In a study reported elsewhere (Cerottini and Brunner, 1967), these A/Sn anti-DBA/2 sera were shown to react strongly in an indirect immunofluorescence test with mastocytoma cells.

In two experiments, equal volumes of either C57BL isoantiserum or control A/Sn isoantiserum were added to reaction mixtures of either sensitized or normal C57BL spleen cells and ^{51}Cr-labelled mastocytoma cells. In both experiments, the C57BL isoantiserum inhibited the cytotoxic effect of sensitized lymphoid cells almost completely, while the control A/Sn antiserum showed no such effect. These results support a specific blocking effect of the C57BL anti-DBA/2 serum.

INHIBITORY EFFECT OF 7S AND 19S ANTIBODY

The effect of individual classes of antibody on the cellular immune reaction was also studied. A hyperimmune isoantiserum was fractionated on a Sephadex G-200 column, and the 7S and 19S classes of globulins separated. The fractions were reconstituted to the original volume, and their haemagglutination titres determined. Mercaptoethanol treatment of the 19S fraction was found to abolish its haemagglutinating activity completely. When added at various dilutions to reaction mixtures, whole serum, 7S and 19S antibody were found to afford, at equivalent titres, similar degrees of protection of target

TABLE 2

INHIBITORY EFFECT OF ANTI-MASTOCYTOMA ISOANTISERUM AND ITS 19S AND 7S FRACTIONS ON THE CYTOTOXICITY OF SENSITIZED LYMPHOID CELLS FOR MASTOCYTOMA TARGET CELLS AS MEASURED BY CLONING ASSAY AND ^{51}Cr RELEASE

Experiment No.	Cytotoxic effect of sensitized lymphoid cells on mastocytoma target cells in the presence of:											
		No antiserum	Antiserum			19S fraction			7S fraction			
1. Measured by cloning assay	Final HA-titre in reaction mixture					1:200	1:70	1:20	1:1,600	1:530	1:160	–	–
	Clones formed by 10^5 target cells after 6 hours of contact with 1×10^7 sens. ly. (counts/min)	1,100				12,300	5,200	2,200	95,700	55,600	10,200	–	–
	with 1×10^7 norm. ly.	120,000				99,000	–	–	111,000	–	–	–	–
	Cytotoxicity (%)	99				88	95	98	14	45	91	–	–
2. Measured by ^{51}Cr release	Final HA-titre in reaction mixture		1:1,600	1:530	1:160	1:80	1:25	–	1:3,200	1:1,070	1:320	1:110	1:30
	Total label incorporated in 10^5 target cells (counts/min)	14,300											
	Label released after 6 hours of contact with 1×10^7 sens. ly. (counts/min)	8,550	4,020	5,380	6,030	5,150	7,530	–	3,230	3,650	4,460	5,800	7,220
	with 1×10^7 norm. ly.	2,090	3,535	3,260	3,200	2,590	2,450	–	3,130	2,630	2,810	2,840	2,470
	Cytotoxicity (%)	52·5	4·5	19	25·5	22	43	–	1	9	14	26	40

cells against the cytotoxic effect of sensitized lymphoid cells. The detailed results of two experiments, in which cytotoxicity was measured by loss of cloning efficiency or by release of radioactive label, are presented in Table 2.

EFFECTS OF MICROSOMAL LIPOPROTEIN ANTIGEN

If isoantibody is effective in blocking antigenic sites at the target cell surface, isoantigen extracted from the target cells might combine with and effectively block hypothetical antibody-like substances at the lymphoid cell surface. H-2 isoantigen preparations

TABLE 3

ANTIGENICITY AND SEROLOGICAL REACTIVITY OF DBA/2 DONOR LIPOPROTEIN ANTIGEN

DBA/2 lipoprotein antigen No.	Protein* (mg/ml)	HA-inhibition titre† (reciprocal)	Cytotoxicity inhibition titre‡ (reciprocal)	C57BL mice injected with 8mg ag. prot. i.p.		Per cent inhibition of *in vitro* enhancing effect¶ of 10 per cent isoantiserum by 1 mg. antigen protein/ml	Inhibition of cytotoxic effects of sensitized spleen cells‖ by 1 mg antigen protein/ml
				HA-titre of serum	Per cent cytotoxicity of spleen cells§		
1	2·5	256	–	–	–	–	0
2	10·0	256	750	<8; 64; 64; 128	0; 6·0; 9·5; 25·7	44·0	0
3	9·0	128	925			88·4/80·0	0/4·3
4	9·5	512	1050			–	0
5	13·0	64	350			–	0
6	14·0	128	450	<8; <8; <8; 16	0; 0; 0; 1·5	–	0

Control antigen: isologous C57BL lipoprotein extracted from kidney tissue was used as a control in all serological reactions at identical concentrations of protein/ml. No inhibitory effect of the control preparation was noted.

* Protein estimation with Folin-reagent standardized with BSA.

† Tested against four HA units of C57BL-anti DBA/2 isoantiserum.

‡ Fifty per cent inhibition of 70–80 per cent lysis of DBA/2 mastocytoma cells by cytotoxic C57BL-anti DBA/2 isoantibody and rabbit complement.

§ Per cent specific ^{51}Cr release by target cells in the presence of 100 sensitized spleen cells per target cell (6 hour incubation). Results of less than 1·5 per cent are listed as 0.

¶ *In vitro* enhancing effect of 10 per cent isoantiserum (HA-titre 1:16,000) expressed as per cent inhibition of specific ^{51}Cr release by target cells in the presence of sensitized lymphoid cells. The results listed express per cent inhibition of the enhancing effect by the addition of 1 mg antigen protein/ml to the reaction mixtures.

‖ Inhibition by 1 mg antigen protein/ml of specific ^{51}Cr release by target cells in the presence of sensitized lymphoid cells. Results of less than 2 per cent are listed as 0.

inducing transplantation immunity and showing serological reactivity have been described by Davies (1966) and by Manson, Dougherty, Foschi and Palm (1964).

A DBA/2 mastocytoma microsomal lipoprotein antigen was, therefore, prepared following essentially the method described by Manson *et al.* (1963). Instead of using the nitrogen decompression method, the cells were broken up by ultrasonication in a nitrogen atmosphere. Lipoprotein preparations were thus obtained with antigenic and serological activity (Table 3), i.e. inhibiting haemagglutination of DBA/2 erythrocytes by C57BL anti-DBA/2 isoantibody, and inhibiting the cytotoxic effect of the same antiserum against donor mastocytoma cells, as measured by ^{51}Cr release (Sanderson, 1964). When

injected into C57BL recipient mice (three intraperitoneal injections of a total of 8 mg antigen protein at 2-day intervals), some of the mice responded with the formation of haemagglutinating antibody at titres reaching 1:128 11 days after the first antigen injection, i.e. titres similar to the ones found in many mice after injection of 3×10^7 living DBA/2 tumour cells. Cellular immunity was only detected in one of these same mice when measured *in vitro*.

When the lipoprotein antigen preparations were tested in the *in vitro* assay of cellular immunity, no inhibitory effect was noted, i.e. the lytic effect of sensitized lymphoid cells was not abolished by the addition of antigen to the reaction mixtures. This shows an apparent lack of reactivity of the lipoprotein antigen for sensitized lymphoid cells.

If isoantibody directed against target cells is able to compete effectively with sensitized lymphoid cells for antigenic sites, the addition of specific antigen preparations to such reaction mixtures should inhibit the blocking effect of isoantibody, and allow target cell lysis by the lymphoid cells. When two such experiments were carried out, the lipoprotein antigen was found to be active, i.e. the *in vitro* enhancing effect of isoantibody was strongly inhibited by addition of antigen. A syngeneic control lipoprotein preparation extracted from kidney tissue of C57BL mice showed no such inhibition when added at equal protein concentrations. Results of these various experiments testing antigenicity and serological reactivity of the lipoprotein antigens are summarized in Table 3.

EFFECT OF DRUGS

To study whether nucleic acid or protein synthesis were necessary for the effect of the immune lymphoid cells, actinomycin-D, an inhibitor known to interfere with DNA-dependent RNA-synthesis, and cycloheximide, an inhibitor of protein (and DNA) synthesis, were added at various concentrations to reaction mixtures of immune or normal spleen cells and target cells. Suspensions containing mastocytoma cells and normal spleen cells in the absence of drugs were included to test for spontaneous release of label and to allow the evaluation of toxicity of the drugs for target cells. No such toxicity was noted for 10 μg/ml actinomycin-D in 6–9 hours, while 10 μg/ml cycloheximide caused a slight increase in release of ^{51}Cr which was corrected for in the evaluation of specific release due to cytotoxic effect of sensitized spleen cells. In parallel experiments, the inhibitory effect of the drugs on the incorporation of [^{3}H]uridine and of [^{14}C]leucine by lymphoid cells was followed. It was found that both actinomycin-D and cycloheximide partially inhibited the cytotoxic effect of sensitized lymphoid cells, as measured by ^{51}Cr release. In seven experiments, drug concentrations of 10 μg/ml, which inhibited the incorporation of the radioactive precursors by 89–96 per cent (uridine) and 85–94 per cent (leucine) inhibited specific liberation of ^{51}Cr from pre-labelled target cells by 21–43 per cent (actinomycin-D) and 31–65 per cent (cycloheximide) (Table 4).

While RNA and protein synthesis appear to be necessary for maximum cytotoxic activity of immune lymphoid cells, it does not necessarily follow that a specific protein necessary for or contributing to the cytotoxic reaction is synthesized. Blocking of the RNA and protein synthesis may lead to profound cellular alterations and thereby indirectly affect the cytotoxic activity of sensitized lymphoid cells. On the other hand, the cytotoxic effect is only reduced by the drug, and not abolished. The lymphoid cells do, therefore, maintain, in the presence of the drug, enough of their cellular integrity to be able to react efficiently with target cells. In addition, if the blocking effect of the drugs

TABLE 4

EFFECT OF DRUGS ON THE INCORPORATION OF LABELLED PRECURSORS BY LYMPHOID CELLS AND ON THE CYTOTOXIC ACTION OF SENSITIZED LYMPHOID CELLS AS MEASURED BY ^{51}Cr RELEASE FROM TARGET CELLS

Experiment No.	Per cent inhibition* by:		Per cent reduction of cytotoxicity†‡ by:	
	Actinomycin (10 μg/ml) of [^{3}H]uridine incorporation	Cycloheximide (10 μg/ml) of [^{14}C]leucine incorporation	Actinomycin (10 μg/ml)	Cycloheximide (10 μg/ml)
1	96	92	43	64
2	89	92	25	38
3	95	94	27	48
4	91	85	21	31
5	93	92	24	65
6	94	91	30	52
7	96	92	37	

* Inhibition of precursor incorporation into lymphoid cells is expressed as: 100—per cent incorporation by suspension in the presence of the drug (as compared to suspensions with no drug added).

† Cytotoxicity expressed as specific release of ^{51}Cr by target cells in the presence of sensitized lymphoid cells (see 'Materials and methods').

‡ Reduction of cytotoxicity is expressed as: 100 minus per cent cytotoxicity of sensitized spleen cells in suspensions containing the drug (as compared to suspensions with no drug added).

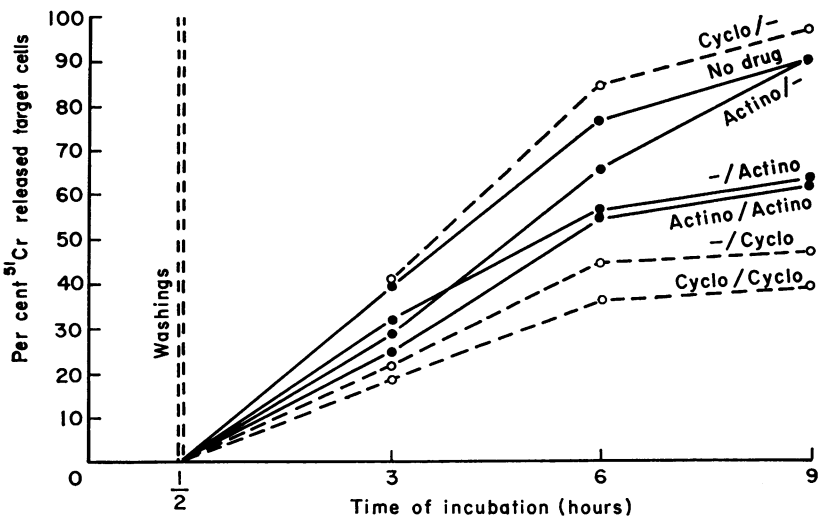

FIG. 3. Inhibitory effect of drugs on target cell lysis by sensitized lymphoid cells. Reversibility of inhibition following removal of drugs by washing. Suspensions of 1×10^{7}/ml sensitized or normal lymphoid cells were pre-incubated for 30 minutes in the presence or absence of 10 μg/ml actinomycin or cycloheximide, followed by washing, addition of labelled target cells, and incubation in the presence or absence of 10 μg/ml of the drugs. For example, pre-incubation in the presence of the drug followed by incubation in the absence of the drug is indicated on the figure by the symbol: drug/–.

was shown to be reversible, this would speak in favour of a specific effect on the synthesis of non-structural protein.

The possible reversibility of the antimetabolic effect of actinomycin-D and of cycloheximide was, therefore, studied. Sensitized lymphoid cells were exposed for 30 minutes to 10 μg/ml of either drug, then washed twice by centrifugation and added to target cells in the presence or absence of drug. Control sensitized and normal spleen cell suspensions were treated similarly in the absence of drugs. In two such experiments, the effect of cycloheximide was completely reversible with regard to the cytotoxic effect on target cells, and almost completely with regard to [^{14}C]leucine uptake. On the other hand, the blocking effect of actinomycin-D was to a large extent irreversible during the first 3 hours incubation, i.e. [H^3]uridine uptake by the drug treated and then washed lymphoid cells was 23 per cent in one experiment (9 per cent in the other) of the uptake of the control cells treated similarly in the absence of drug, and cytotoxicity remained close to the level

TABLE 5

REVERSIBILITY OF DRUG EFFECT ON THE INCORPORATION OF RADIOACTIVE PRECURSORS BY LYMPHOID CELLS

Precursor added after pre-incubation and washing	Drug (act. or cyclo.) added before/after washing	Incorporation of precursor by lymphocytes following: 30 minutes pre-incubation				3 hours pre-incubation			
		Experiment 1		Experiment 2		Experiment 1		Experiment 2	
		Counts/min	Per cent	Counts/min	Per cent	Counts/min	Per cent	Counts/min	Per cent
[^{3}H]Uridine	-/-	47,798	100	52,160	100	50,873	100	35,721	100
	Act./-	11,234	23	4,823	9	911	2	606	2
	Act./Act.	2,354	5	2,110	4	–	–	374	1
[^{14}C]Leucine	-/-	5,857	100	13,525	100	3,061	100	4,267	100
	Cyclo/-	6,127	100	12,281	91	2,031	66	3,459	81
	Cyclo/Cyclo	376	6	997	7	–	–	912	24

Spleen cell suspensions of 1×10^7 cells/ml are pre-incubated for 30 minutes or 3 hours in the presence or absence of 10 μg/ml actinomycin or cycloheximide, followed by washing and incubation for 3 hours in the presence or absence of 10 μg/ml of the drugs. Radioactive precursor added after washing.

of cells in the presence of the drug. While the continued presence of actinomycin increasingly depressed the cytotoxic activity for up to 9 hours incubation, the lymphocytes washed free of the drug appeared to recover, with the rate of target cell lysis approximating the controls. Detailed results of one of the experiments are presented in Fig. 3.

Reversibility of the drug effect on the incorporation of radioactive precursors by spleen cells was tested for in several additional experiments, with drug removal by washing after 30 minutes or 3 hours. As shown in Table 5, similar results were obtained, confirming reversibility of the blocking effect of cycloheximide even after 3 hours exposure before washing, and irreversible effect of actinomycin-D at least for a 3-hour incubation period.

DISCUSSION

The results presented demonstrate that spleen cells of mice sensitized by a tumour allograft destroy donor target cells *in vitro* within 6–9 hours, as indicated by specific ^{51}Cr release and microscopic observations. The cytotoxic effect of immune spleen cells appears

to involve a mechanism of increased permeability of the cell wall leading to cellular disintegration. It was, therefore, decided to use the term 'lysis' in describing the cytotoxic effect of sensitized lymphoid cells as measured by specific release of radioactive ^{51}Cr.

Lysis is shown to depend on the number of sensitized lymphoid cells in the reaction mixture, and to proceed linearly over most of its time course. Target cell inactivation as measured by growth inhibition (cloning efficiency) proceeds at similar rates.

Normal allogeneic spleen cells did not affect target cells under the conditions of the tests. This apparent lack of allogeneic inhibition (Hellström and Hellström, 1965; Möller and Möller, 1965) is presumably based on the fact that intact allogeneic cells do not, in the absence of stimulation and/or aggregation by antibody or phytohaemagglutinin, adversely affect target cells *in vitro*.

Multiples of 100 lymphocytes per mastocytoma cell are shown by the present studies to reduce cloning efficiency of the target cells by up to 99 per cent within 6 hours, while up to 90 per cent of the radioactive label incorporated was released within the same time period. Under the conditions of the experiments using stationary Leighton tubes, microscopic observation of reaction mixtures showed that not more than ten lymphocytes were close enough to one target cell to effect an intimate contact. While most of the mastocytoma cells had one or at most a few lymphoid cells attached to their surface, lymphocytes from non-immune control mice also occasionally stuck to target cells; one could not observe a striking clustering of sensitized cells around target cells. On the other hand, the results of Wilson (1965a) and our own experiments (Brunner *et al.*, 1966) suggest that target cell inactivation by sensitized lymphoid cells is based on a mechanism similar to a single hit phenomenon, in which one lymphoid cell is sufficient to destroy or damage detectably one target cell. For complete inactivation, the number of lymphoid cells able to attack mastocytoma cells should therefore be at least equal to the number of target cells, i.e. at a ratio of 100 lymphocytes per target cell, 1 per cent of the spleen cell population would be expected to be sensitized. In experiments aimed to evaluate the number of antibody producing cells in the spleen of animals sensitized by one injection of sheep red cells (Jerne, Nordin and Henry, 1963) it has been shown that this proportion is lower than 0·1 per cent. It seems, therefore, necessary that a proportion of cells far above that producing detectable extracellular antibodies is active in terms of 'cellular immunity'. Alternatively, a mediator substance may be liberated by some of the spleen cells, perhaps after stimulation by contact with antigen, which then acts directly or indirectly on the target cells. Such a substance has been demonstrated in a delayed hypersensitivity reaction *in vitro* by Bloom and Bennett (1966), a test which similarly requires the presence of only a small percentage of sensitive cells in the population of migrating cells, and which has been shown to be applicable to homotransplantation systems (Al-Askari, David, Lawrence and Thomas, 1965).

The results demonstrating a blocking effect of isoantibody confirm earlier studies of Möller (1965) and Brunner *et al.* (1967a, b), and are in support of the concept of an efferent mechanism of immunological enhancement (Billingham, Brent and Medawar, 1956). Efferent inhibition by 19S antibody is a phenomenon which is presumably restricted to *in vitro* reactions, since under *in vivo* conditions, 19S antibody would fix complement and induce target cell lysis.

A slight cytotoxicity of the isoantiserum or its 7S and 19S fractions for target cells was almost regularly encountered. This might be due to inadequate inactivation of the calf serum present in the culture medium, to small amounts of complement formed by the

cells themselves (Thorbecke, Hochwald, Furth, Müller-Eberhard and Jacobson, 1965), or to a direct action of the antibody, which has been shown by Bitensky (1963) to cause permeabilization of lysosomal membranes in the absence of complement.

If inhibition by isoantiserum of the cytotoxic effect of sensitized lymphoid cells is due to competition for the same antigenic determinants, lack of inhibition of cell-mediated cytotoxicity by antigenic extracts would suggest that the specific antigenic reacting site has been lost during the course of extraction. The antigenic extract might then be considered as acting like a partial antigen, capable of fixing antibodies, while reaction with sensitized cells would require the whole antigenic histocompatibility complex. This would also agree with the concept that delayed hypersensitivity is concerned with broader antigenic determinants than circulating antibody.

The results demonstrating that blocking of protein synthesis (or of DNA-dependent RNA synthesis) reduces the cytotoxic effect of sensitized lymphoid cells are in agreement with the concept that cellular transplantation immunity *in vitro* is dependent on protein synthesis. Full reversibility of the blocking effect of cycloheximide indicates that the structural integrity of the cells is not affected, and suggests that the chemical inhibits the synthesis of non-structural protein. In addition, the quantitative analysis of the drug effect allowed the demonstration that the cytotoxic activity of sensitized lymphoid cells is partially drug resistant. The lytic activity in the presence of drug concentrations which reduced RNA and protein synthesis to values of 10 per cent of control spleen cell suspensions might be explained by preformed pools in the sensitized cells.

The experiments described in this study were based on an assay system directly measuring target cell inactivation as a function of release of radioactive label. Such a system possesses several advantages. It is technically simple, and provides accurate, quantitative results within short time periods. It is independent of target cell multiplication, and thus readily allows the study of metabolic inhibitors. It is applicable to any target cell type which can be radioactively labelled, and thus should be of considerable help in further studies of the mechanism of lymphocyte–target cell interaction, as well as in the study of tumour immunity.

ACKNOWLEDGMENT

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

REFERENCES

Al-Askari, S., David, J. R., Lawrence, H. S. and Thomas, L. (1965). '*In vitro* studies on homograft sensitivity.' *Nature* (*Lond.*), **205**, 916.

Amos, D. B. (1962). 'Isoantigens of mouse red cells.' *Ann. N.Y. Acad. Sci.*, **97**, 69.

Billingham, R. E., Brent, L. and Medawar, P. B. (1956). 'Enhancement in normal homografts, with a note on its possible mechanism.' *Transplant. Bull.*, **3**, 84.

Bitensky, L. (1963). 'Cytotoxic action of antibodies.' *Brit. med. Bull.*, **19**, 241.

Bloom, B. R. and Bennett, B. (1966). 'Mechanism of a reaction *in vitro* associated with delayed-type hypersensitivity.' *Science*, **153**, 80.

Brunner, K. T., Mauel, J. and Schindler, R. (1966). '*In vitro* studies of cell-bound immunity; cloning assay of the cytotoxic action of sensitized lymphoid cells on allogeneic target cells.' *Immunology*, **11**, 499.

Brunner, K. T., Mauel, J. and Schindler, R. (1967a). '*In vitro* studies of cell-bound immunity induced by tumor allografts; inhibitory effects of isoantibody.' *Germinal Centers in Immune Responses*, p. 297. Springer-Verlag, Berlin.

Brunner, K. T., Mauel, J. and Schindler, R. (1967b). 'Inhibitory effect of isoantibody on *in vivo* sensitization and on the *in vitro* cytotoxic action of immune lymphocytes.' *Nature* (*Lond.*), **213**, 1246.

Calabresi, P., Brunner, K. T. and Mauel, J. (1967). 'Inhibition by puromycin *in vitro* of cell-bound immunity by tumor allografts.' *Fed. Proc.*, **26**, 478 (Abstract 1233).

Cerottini, J.-C. and Brunner, K. T. (1967). 'Localization of mouse isoantigens on the cell surface as revealed by immunofluorescence.' *Immunology*, **13**, 395.

David, J. R. (1965). 'Suppression of delayed hypersensitivity *in vitro* by inhibition of protein synthesis.' *J. exp. Med.*, **122**, 1125.

DAVIES, D. A. L. (1966). 'Mouse histocompatibility antigens derived from normal and from tumour cells.' *Immunology*, **11**, 115.

DUNN, T. B. and POTTER, M. (1957). 'A transplantable mast-cell neoplasm in the mouse.' *J. nat. Cancer Inst.*, **18**, 587.

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

HELLSTRÖM, K. E. and HELLSTRÖM, I. (1965). 'Syngeneic preference and allogeneic inhibition.' *Isoantigens and Cell interactions*, p. 79. The Wistar Institute Press, Philadelphia.

JERNE, N. K., NORDIN, A. A. and HENRY, C. (1963). 'The agar plaque technique for recognizing antibody producing cells.' *Cell-bound Antibodies*, p. 109. The Wistar Institute Press, Philadelphia.

MANSON, L. A., DOUGHERTY, T., FOSCHI, G. V. and PALM, J. (1964). 'Microsomal lipoproteins as transplantation antigens.' *Ann. N.Y. Acad. Sci.*, **120**, 251.

MANSON, L. A., FOSCHI, G. V. and PALM, J. (1963). 'An association of transplantation antigens with microsomal lipoprotein of normal and malignant cells.' *J. comp. Physiol.*, **61**, 109.

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

MÖLLER, G. and MÖLLER, E. (1965). 'The cytotoxic *in vitro*.' *Isoantigens and Cell Interactions*, p. 65. The Wistar Institute Press, Philadelphia.

ROSENAU, W. (1963). 'Interaction of lymphoid cells with target cells in tissue culture.' *Cell-Bound Antibodies*, p. 75. The Wistar Institute Press, Philadelphia.

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

SANDERSON, A. R. (1964). 'Cytotoxic reactions of mouse isoantisera: preliminary considerations.' *Brit. J. exp. Path.*, **45**, 398.

STIMPFLING, J. H. (1961). 'Use of PVP as developing agent in mouse hemagglutination tests.' *Transplant Bull.*, **27**, 109.

THORBECKE, G. J., HOCHWALD, G. M., VAN FURTH, R., MÜLLER-EBERHARD, H. J. and JACOBSON, E. B. (1965). 'Problems in determining the sites of synthesis of complement components.' *Ciba Foundation Symposium*: *'Complement'* (Ed. by G. E. W. Wolstenholme and J. Knight), p. 99. Churchill, London.

VAINIO, T., KOSKIMIES, O., PERLMANN, P., PERLMANN, H. and KLEIN, G. (1964). '*In vitro* cytotoxic effect of lymphoid cells from mice immunized with allogeneic tissue.' *Nature (Lond.)*, **204**, 453.

WILSON, D. B. (1965a). 'Quantitative studies on the behavior 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 effect of isoimmune serum.' *J. exp. Med.*, **122**, 143.

WILSON, D. B. (1965b). 'Quantitative studies on the behavior 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.