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INDUCTION AND SUPPRESSION OF THE CYTOTOXIC ACTIVITY OF HUMAN LYMPHOCYTES *IN VITRO* BY HETEROLOGOUS ANTI-LYMPHOCYTE SERUM

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SUMMARY

Heterologous anti-lymphocyte sera were demonstrated to induce a cytotoxic potential in normal non-immunized human lymphocytes against allogeneic fibroblast target cells. The cytotoxicity-inducing capacity was restricted to certain dilutions of anti-lymphocytic serum above and below which no cytotoxic effect was obtained. This optimal concentration shifted towards higher dilutions in sera taken late during the immunization course. The antisera were shown to stimulate the DNA-synthesis in lymphocytes and to aggregate the lymphocytes to the target cells. The DNA-synthesis and the aggregation as well were maximal at the same dilution of anti-lymphocytic serum which induced cytotoxicity. No cytotoxic effect was demonstrable on sheep fibroblasts. It is, therefore, suggested that the anti-lymphocytic serum antibody induces lymphocyte-mediated cytotoxicity against allogeneic fibroblasts in a two step manner: it stimulates the lymphocytes into a cytotoxic state; it aggregates the human lymphocytes to the human fibroblasts by virtue of its bivalent structure.

Anti-lymphocytic serum was also found to suppress the cytotoxic activity of lymphocytes induced by various non-specific agents, such as phytohaemagglutinin, streptolysin O and anti-lymphocytic serum itself. The mechanism for this inhibition is extensively discussed and it is suggested that anti-lymphocytic serum suppresses the reaction by coating the lymphocytes, thereby preventing the intimate contact between effector and target cell. A similar mechanism may operate *in vivo* and could be a partial explanation of the *in vivo* immunosuppressive effect of anti-lymphocytic serum. Purified 7S γ-globulin possessed all activities of the whole antiserum.

INTRODUCTION

In a previous publication (Lundgren & Möller, 1969) the cytotoxic effect of non-immunized human lymphocytes was investigated in an *in vitro* system where the lymphocytes were

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applied to fibroblast monolayers of different genotypes in the presence of phytohaemagglutinin (PHA). A cytotoxic effect could be detected 4–8 hr after the addition of PHA and developed equally well on autochthonous and on allogeneic target cells. It was suggested, therefore, that expression of cytotoxicity is an immunologically non-specific process caused by stimulated lymphocytes, and that the specificity of cellular immune reactions is confined to the immunological recognition step initiating the cytotoxic potential of the lymphocytes. According to this two step concept, specific recognition would be by-passed by stimulation with PHA. Living lymphocytes and a close contact between effector and target cells were required for cytotoxicity to occur. Actinomycin-C, which is known to suppress the DNA-dependent RNA-synthesis (Reich *et al.* 1961) did not reduce the cytotoxic effect of the PHA-treated lymphocytes. Similar negative results were obtained with Mitomycin-C and X-irradiation, suggesting that the cytotoxic potential of the lymphocytes developed independently of other expressions of PHA-stimulation, such as increased RNA- and DNA-synthesis and morphological transformation.

The present study concerns the effect of heterologous anti-lymphocyte serum (ALS) in this system. As will be shown below ALS can suppress the PHA-induced cytotoxicity of human lymphocytes on fibroblast target cells. Since ALS has been shown to be a powerful immunosuppressive agent *in vivo* (Woodruff & Anderson, 1963; Monaco *et al.*, 1967; Starzl *et al.*, 1967; Traeger *et al.*, 1969) it seemed possible that this inhibition could be used as an *in vitro* method to evaluate the immunosuppressive capacity of an ALS. Therefore, particular attention was focused on the mechanism of the ALS-induced suppression of lymphocyte-mediated cytotoxicity.

MATERIALS AND METHODS

Human and sheep fibroblast monolayers and human peripheral blood *lymphocytes* were obtained and cultivated as previously described (Möller, Beckman & Lundgren, 1966; Möller *et al.*, 1967; Lundgren, Zukoski & Möller, 1968b; Lundgren & Möller, 1969). Contaminating granulocytes were removed by carbonyl iron powder (Lundgren *et al.*, 1968b) and not more than 1% granulocytes in the lymphocyte suspension was allowed.

The cellular synthesis of DNA in the lymphocytes was measured by incorporation of [^{14}C]thymidine (Amersham, England, specific activity 25 mCi/mM) as described (Lundgren & Möller, 1969). The precursor was added to the test culture after 48 hr, and the degree of incorporation was measured during a 24-hr period.

Heterologous anti-lymphocyte serum

The antiserum was prepared by repeated injections of human spleen cells and peripheral blood cells, respectively, into a horse. The immunization schedule and types of cells injected are presented in Fig. 1. The spleens were removed aseptically from recently deceased humans and placed in cold Ringer solution. They were then cut in small pieces and pressed through a 60 mesh stainless steel screen. The cells were suspended in Ringer's solution and filtered through gauze. To lyse most red cells the cells were spun down and the supernatant replaced by distilled water. One minute later an equal volume of 1·8% saline was added, the cells washed twice in Ringer's solution and finally suspended in a volume of Ringer's solution convenient for injection into the horse. All spleen cell suspensions were injected subcutaneously and the cells from one spleen were used for each immunization. Peripheral blood

leucocytes were obtained from blood donors. For each immunization 400 ml blood was taken from each of twenty to thirty persons. The leucocytes were isolated from the pooled 'buffy coats' from the blood samples. To lyse the erythrocytes the cells were suspended in 0·83% ammonium chloride solution (Strander & Cantell, 1966) and placed in the refrigerator (+4°C) for 20 min, whereafter the cells were spun down and the supernatant discarded. This procedure was repeated three times. The cells were finally suspended in Ringer's solution and injected intravenously into the horse. The final cell suspension was almost free from contaminating erythrocytes.

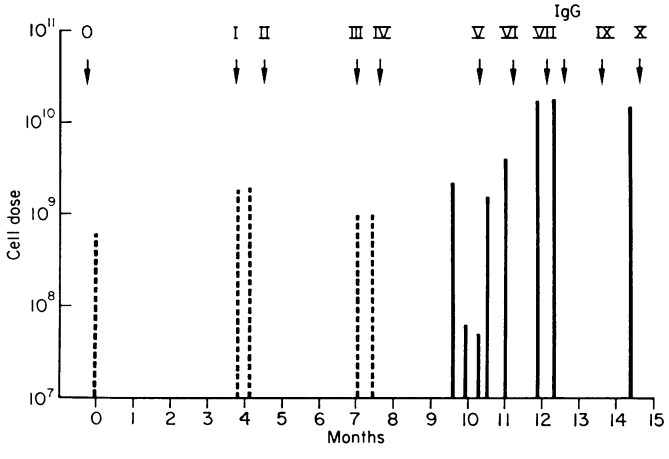

FIG. 1. Immunizing schedule of the horse producing the anti-lymphocytic sera. Spleen cells (broken columns) were given subcutanously and peripheral blood leucocytes (solid columns) were given intravenously. When the different ALS batches were taken is indicated on top of the chart.

The horse was bled at various intervals and the serum was collected after clotting. Thus, different batches of antiserum were obtained and they were labelled as indicated in Fig. 1. From one batch (IgG) the 7S fraction of the γ-globulin was isolated using DEAE-Sephadex according to the method described by Perper *et al.* (1967). The final product contained 5 g protein/100 ml and no 19S γ-globulin as demonstrated by immunoelectrophoresis. All sera were heated to 56°C for 30 min before use.

Cytotoxic and lymphoagglutinating titres

The cytotoxic titre of an antiserum was determined as follows: The antiserum was serially diluted ($\log_2$) in Ringer's solution and an equal volume (0·05 ml) of a lymphocyte suspension containing 5×10^6 cells/ml was added to each tube. The mixture was incubated in a water bath at 37°C for 30 min, whereafter 0·05 ml of rabbit serum diluted 1:2 was added as a complement source. After shaking, the tubes were reincubated in a water bath for another 60 min. The supernatant was then carefully removed and the cells suspended in 0·15 ml of a 0·16% trypan blue solution and the percentage of trypan blue excluding cells was immediately determined in the microscope. The cytotoxic titre was defined as the highest antiserum dilution where 50% of the lymphocytes were killed.

The lymphoagglutinating titre was determined by diluting the antiserum in 1·5% polyvinylpyrrolidone (PVP) followed by addition of an equal volume (0·05 ml) of a lymphocyte

suspension containing 5×10^6 cells/ml. After incubation in a water bath at 37°C for 90 min the cells were resuspended and the entire volume in the test tube placed on a microscope slide. When the cells had settled down on the glass the degree of lympho-agglutination was determined. The lympho-agglutinating titre was defined as the highest serum dilution where some aggregates of more than two to three lymphocytes could be seen.

Lymphocyte-mediated cytotoxicity on fibroblasts

One-tenth of a millilitre of a lymphocyte suspension containing 10^4–10^6 cells was added to pre-indicated sites on fibroblast monolayers. One to four sites were used per Petri dish (50 mm in diameter). The cells were allowed to settle down on the monolayer for at least 30 min before any stimulating agent was added to the culture medium. The dishes were cultivated, harvested, fixed and stained as previously reported (Möller *et al.*, 1967). Destruction (plaque-formation) of the monolayer was scored with the naked eye or under the microscope and graded from 0 to 4+. 1+ indicated a visible plaque without complete destruction of the monolayer and 4+ a complete destruction of the monolayer in an area with a diameter of more than 1·5 cm.

Drugs and chemicals

Phytohaemagglutinin (Wellcome Foundation, England) was delivered as a freeze-dried material which was reconstituted with 5 ml of sterile distilled water. This stock solution was used in a final concentration of 1:100, which was the optimal dose for stimulation of [^{14}C]-thymidine incorporation. Streptolysin O (State Bacteriological Laboratory, Sweden) contained 6–10 streptolysin units/ml and was used in concentrations of 1:10 to 1:100.

RESULTS

Various anti-lymphocyte sera were capable of transforming lymphocytes into a cytotoxic state expressed on human fibroblasts by the destruction of the monolayer at the area of lymphocyte application. As can be seen in Table 1 the induction of lymphocyte-mediated target destruction was restricted to certain concentrations of ALS. An optimal concentration capable of inducing cytotoxicity was obtained for each ALS, although this optimum could vary one to two dilution steps between different experiments (Table 1). At higher or lower concentrations of ALS no damage to the fibroblasts could be revealed. The optimal concentration inducing cytotoxicity shifted towards higher dilutions in antisera taken later during the immunization course. The cytotoxic effect induced by ALS seldom exceeded 1+ to 2+ and was rarely achieved with less than 10^6 lymphocytes. A weak (1+) reaction was obtained with 10^5 lymphocytes in five experiments out of twenty-one. ALS did not have to be present continuously in the culture medium for cytotoxicity to occur. Thus, pre-treatment of the lymphocytes with ALS diluted 1:10 to 1:200 for 3 days in tissue culture tubes (the lymphocytes being cultivated in Eagle's suspension medium supplemented with 10% human heat-inactivated AB-serum) followed by three washings in Ringer's solution prior to their application to the fibroblasts was sufficient to cause a cytotoxic damage to the fibroblast monolayer (Table 2). Damage caused by such lymphocytes was more pronounced in many experiments than when ALS was continuously present in the culture medium.

The cytotoxic effect exerted by lymphocytes in the presence of ALS and by ALS-pre-treated lymphocytes as well was abolished by heating the lymphocytes to 48·5°C for 30 min

TABLE 1. Lymphocyte cytotoxicity on human fibroblasts induced by various dilutions of different ALS batches in relation to their toxic effect on lymphocytes in the absence of complement and their capacity to aggregate lymphocytes to the target cells

ALS batch	Parameter studied	Experiment No.	Dilution of ALS 1:10	1:50	1:100	1:200	1:400	1:800	1:1600
I	Plaque-formation*	1	0	0	0	0	0		
II	Plaque-formation	1	+++						
		2	+++		+				
		3	+		+				
		4		+	++				
		5		+	+	0	0	0	
	Lymphocyte-target aggregation†			Max	Max				
	Toxicity index‡ on lymphocytes		86	91	93	90	85	81	
IV	Plaque-formation	1	+						
		2	+++	+					
		3	0	++	+++				
		4	0	+	+	0	0		
		5	0	0	0	0	0		
	Lymphocyte-target aggregation			Max					
	Toxicity index on lymphocytes		95	87	90	85	65	59	
VII	Plaque-formation	1	0	0	0	+			
		2		0	0	+	+	0	
		3		0	+	+	0	0	
		4		0	0	+	+	+	
	Lymphocyte-target aggregation					Max	Max		
	Toxicity index on lymphocytes		87	89	89	81	69	82	
X	Plaque-formation	1		0	0	0	+	+	
	Lymphocyte-target aggregation						Max		
	Toxicity index on lymphocytes		92	85	80	78	60	68	
IgG	Plaque-formation	1		0	0	+	+	+	+
	Lymphocyte-target aggregation							Max	Max
	Toxicity index on lymphocytes			68	72	68	58	52	

* Cytotoxicity (plaque-formation) was graded between 0 and 4+ and the cultures were read after 6 days. One million lymphocytes were added per application site.

† After 3 days culture at various concentrations of ALS the dishes were washed, fixed and stained. The dilution of ALS which had the maximal capacity to aggregate lymphocytes to the target cells was determined under the microscope and marked 'Max' in the table.

‡ The toxicity of ALS on lymphocytes in the absence of complement was based upon counting the number of surviving lymphocytes after 3 days culture in the presence or absence of ALS according to the formula $(a-b/a)\times 100$ where a = proportion of trypan blue excluding cells in cultures without ALS, and b = proportion of trypan bue excluding cells in cultures with ALS.

prior to their addition to the fibroblast cultures. Thus, active lymphocyte reactions appear to be necessary for cytotoxicity to occur. The killing effect of ALS-stimulated lymphocytes could only be demonstrated on human fibroblasts. Using sheep fibroblasts none of the ALS preparations used at any concentrations was able to render the lymphocytes cytotoxic against the targets. Lymphocytes pre-treated with ALS for 2–3 days were equally inefficient on sheep fibroblasts (Table 2).

TABLE 2. Effect of pre-treating human lymphocytes with ALS for 2–3 days on cytotoxicity against human and sheep fibroblasts, respectively (cultures were read after 6 days)

Lymphocyte donor	ALS batch	Pre-treatment dose	Target cells	Plaque-formation
GC	IV	1:10	RF (human)	+++
			Sheep	0
BS	VI	1:10	KL (human)	+++
			Sheep	0
ML	VI	1:10	KL (human)	+++
			Sheep	0
JF	VI	1:10	KL (human)	++
			Sheep	0
ÅM	VII	1:200	RF (human)	+
			Sheep	0

ALS was found to aggregate the lymphocytes to the target cells. Maximal aggregation was achieved at a certain concentration of ALS, above and below which aggregation was less pronounced. The dilutions of the various antisera which caused the strongest aggregation are indicated in Table 1. These dilutions corresponded to those which induced a maximal lymphocyte-mediated cytotoxicity.

ALS was toxic to the lymphocytes. At certain concentrations of some ALS-batches only 10% of the lymphocytes survived 3 days culture. In spite of this fact cytotoxicity occurred. No correlation was found between the toxic effect on the lymphocytes and failure to induce cytotoxicity.

Stimulation of DNA-synthesis by various anti-lymphocyte sera

Since ALS is known to stimulate synthetic processes in the lymphocytes (Gräsbeck, Nordman & De la Chapelle, 1963) such as RNA, DNA and protein synthesis, the stimulating capacities of the different sera were estimated by measuring the incorporation of [^{14}C] thymidine into the lymphocytes. All antisera were tested on the same day using lymphocytes from one single donor. Maximal stimulation occurred after 2–3 days. For each ALS there was an optimally stimulating concentration, higher and lower concentrations giving a lower degree of stimulation. Except for the early antisera, all sera failed to stimulate at dilution 1:10 or lower and at 1:800 or higher, disregarding the optimally stimulating concentration and the intensity of stimulation. Thus, they were stimulating only over an eighty-fold range of dilutions. As can be seen from Fig. 2 the maximally stimulating concentration of ALS was shifted towards more diluted sera as immunization proceeded. The

optimally stimulating concentration for the different ALS preparations was also optimal for induction of lymphocyte-mediated cytotoxicity (compare Table 1). However, no correlation existed between the actual degree of stimulation (measured in counts/min) and the cytotoxicity-inducing effect of the antisera at various concentrations. No ALS had a cytotoxicity-inducing capacity as strong as PHA, although many of them stimulated DNA-synthesis to a higher extent than PHA (Fig. 2).

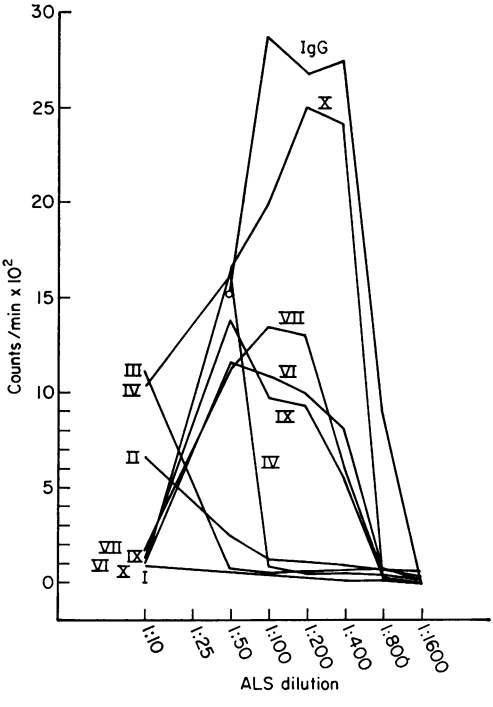

FIG. 2. [^{14}C]Thymidine incorporation (measured in counts/min) into human lymphocytes cultured with various ALS batches at the indicated dilutions. [^{14}C]Thymidine was added after 48 hr and the cultures harvested 24 hr later. Each point on the graph represents the mean value of triplicate cultures. [^{14}C]Thymidine incorporation into lymphocyte cultures in the presence of phytohaemagglutinin (1:100) in parallel cultures was $7{\cdot}08 \times 10^2$ counts/min.

Inhibition of PHA-induced lymphocyte-mediated cytotoxicity by ALS

Lundgren & Möller (1969) investigated the PHA-induced cytotoxic effect of human lymphocytes against fibroblast target cells. The *in vitro* system used may represent a model of the efferent pathway of the allograft reaction *in vivo* and therefore it was considered to be of interest to evaluate the effect of ALS on this PHA-induced lymphocyte-mediated cytotoxicity, since ALS is known to have immunosuppressive properties *in vivo*. Therefore, ALS at various concentrations was added to the culture after application of the lymphocytes and PHA. It was found that ALS efficiently suppressed plaque-formation on human fibroblasts (Fig. 3). Using antisera taken early during the immunization course (ALS II and IV) complete suppression was not obtained even with high concentrations of ALS, but as can be seen in Table 1 these antisera by themselves induced lymphocyte-mediated cytotoxicity at concentrations of 1:10 to 1:100. ALS VII and X obtained late in immunization and which

induced lymphocyte-mediated cytotoxicity by themselves only at concentrations of 1:200 to 1:800 completely abolished PHA-induced cytotoxicity of lymphocytes at concentrations higher than 1:200. A partial suppression of plaque-formation was achieved with all antisera in dilutions up to 1:100 to 1:800.

Since ALS is a heterologous antiserum directed against human tissues its inhibiting effect on the PHA-induced cytotoxic effect of human lymphocytes on human fibroblasts could

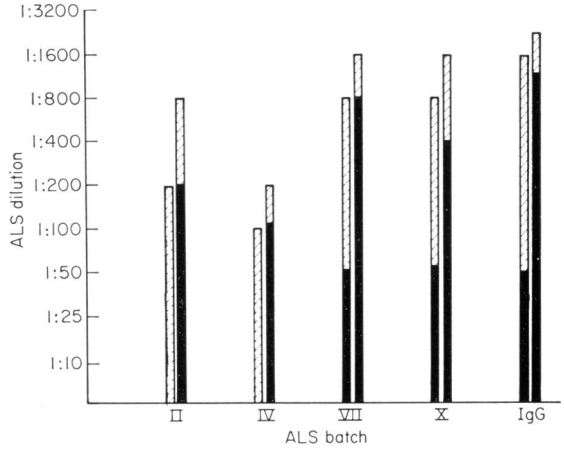

FIG. 3. Inhibition of PHA-induced lymphocyte cytotoxicity on human (left column) and sheep (right column) fibroblasts, respectively, by indicated dilutions of various anti-lymphocyte sera. Broken columns represent partial inhibition, solid columns represent total inhibition.

be due to an effect on the lymphocytes and/or on the fibroblasts. Suppression could conceivably also be due to an inactivating effect of PHA by ALS. To test the possibility that ALS adheres to and covers the fibroblasts, thereby protecting them from the cytotoxic action of the PHA-stimulated lymphocytes, sheep fibroblasts were used as targets. Before use the antisera were absorbed with sheep red blood cells (SRBC). After this no haemagglutinins or haemolysins (tested in the presence of rabbit or guinea-pig complement) could be demonstrated against SRBC indicating that the antisera did not contain any antibodies against sheep tissue cells. However, the antisera suppressed PHA-induced plaque-formation on sheep fibroblasts even more efficiently than when tested on human targets (Fig. 3). Pre-treatment of the lymphocytes with ALS for 3 days was sufficient to suppress plaque-formation on sheep fibroblasts in the presence of PHA, in contrast to analogous experiments performed on human fibroblasts (Table 3). The inhibiting effect of ALS, therefore, cannot be due to a blocking effect of the targets protecting them from destruction. To investigate if ALS inactivates PHA, lymphocytes were pre-treated with PHA for 3 days and then washed carefully three times in Ringer's solution before application to the fibroblasts in the presence of ALS. Since ALS also in this system suppressed plaque-formation by the pre-treated already cytotoxically activated lymphocytes (Table 4), it was concluded that the inhibiting effect of ALS was not caused by inactivation of the stimulating effect of PHA, but rather by an action on the lymphocytes.

Maximal suppression of PHA-induced plaque-formation by human lymphocytes on human fibroblasts was obtained by adding the ALS within 4 hr after PHA. If ALS was

added later it was less efficient, indicating that the PHA-induced cytotoxic effect of lymphocytes was initiated after 4 hr. On sheep fibroblasts plaque-formation was completely suppressed by adding ALS up to 12 hr after PHA in some experiments.

The horse serum was purified as described in 'Materials and methods' so that a pure 7S γ-globulin fraction was received (batch IgG). As can be seen in Figs. 2 and 3 and Table 1 the 7S antibody was fully capable to stimulate DNA-synthesis in the lymphocytes, to transform them into a cytotoxically active state, and to suppress the cytotoxic effect of lymphocytes induced by PHA.

TABLE 3. Effect of ALS-pre-treatment of human lymphocytes on their ability to cause plaque-formation on human and sheep fibroblasts, respectively, in the presence of PHA

Lymphocyte dose	Pre-treatment with:	Target cells	PHA 1:100 in medium	Plaque-formation at day		
				2	4	6
10^6	Medium	Human	−	0	0	0
	Medium	Human	+	+++	+++	+++
	ALS IV 1:10	Human	−	++	+	++
	ALS IV 1:10	Human	+	+++	+++	+++
10^5	Medium	Human	−	0	0	0
	Medium	Human	+	+	++	++
	ALS IV 1:10	Human	−	0	+	+
	ALS IV 1:10	Human	+	+	++	+++
10^6	Medium	Sheep	−	0	0	0
	Medium	Sheep	+	++++	++++	++++
	ALS VII 1:200	Sheep	−	0	0	0
	ALS VII 1:200	Sheep	+	+	+	+

Morphology

PHA caused a strong aggregation of the lymphocytes to the fibroblast target cells which were finally almost covered by lymphocytes (Fig. 4). ALS in high concentrations changed this aggregation pattern. Thus, the lymphocytes agglutinated to each other in large aggregates, which were easily removed by washing, and bound less efficiently to the target cells (Fig. 5). However, when diluted, ALS caused a pronounced lymphocyte-target aggregation (Fig. 6) and the lymphocytes became morphologically transformed. The dilutions of ALS which transformed the lymphocytes and aggregated them to the targets corresponded to those which rendered the lymphocytes cytotoxic (see above). On sheep fibroblasts no lymphocyte–target cell aggregation was obtained with ALS at any dilution and the lymphocytes were easy to wash away.

Cytotoxic and lymphoagglutinating titres

The cytotoxic and the lymphoagglutinating titres of all antisera were determined on the same day using lymphocytes from the same donor as target cells. In Fig. 7 these titres have been plotted together with the titres at which the different antisera caused a total and partial inhibition, respectively, of the PHA-induced cytotoxic effect of lymphocytes on sheep fibroblasts. As can be seen in Fig. 7 there was a comparatively good parallelism between

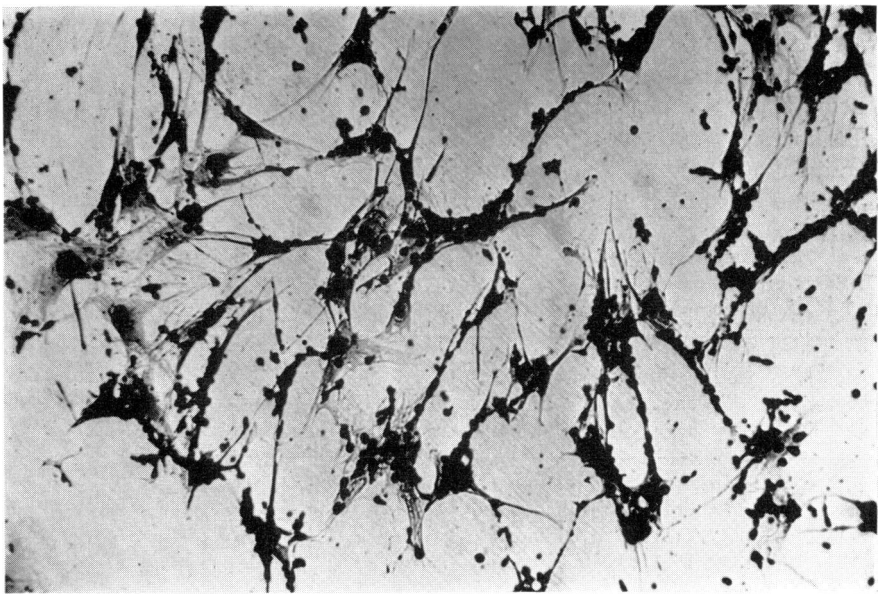

FIG. 4. Lymphocyte–target cell aggregation 6 hr after addition of phytohaemagglutinin to the culture medium.

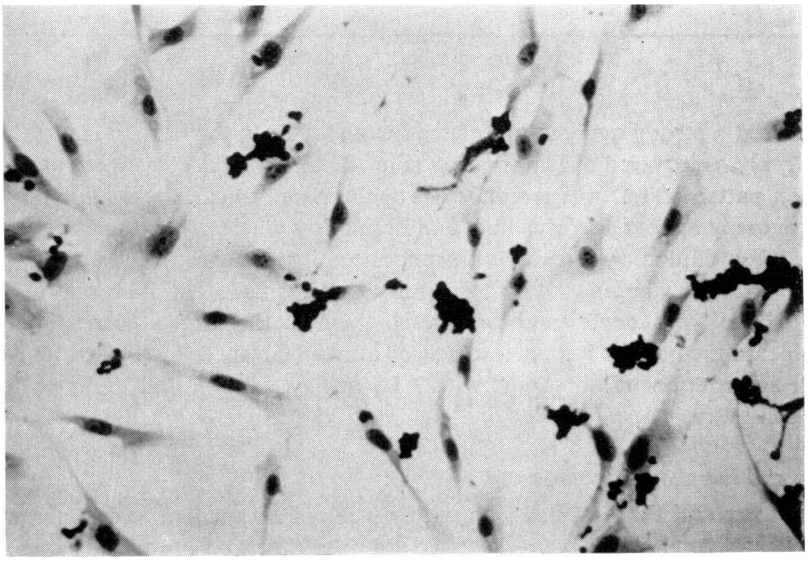

FIG. 5. Lymphocyte–target cell aggregation 8 hr after addition of PHA and ALS IV (1 : 50) to the culture medium. After 6 hr hardly any lymphocyte–target cell aggregation could be demonstrated.

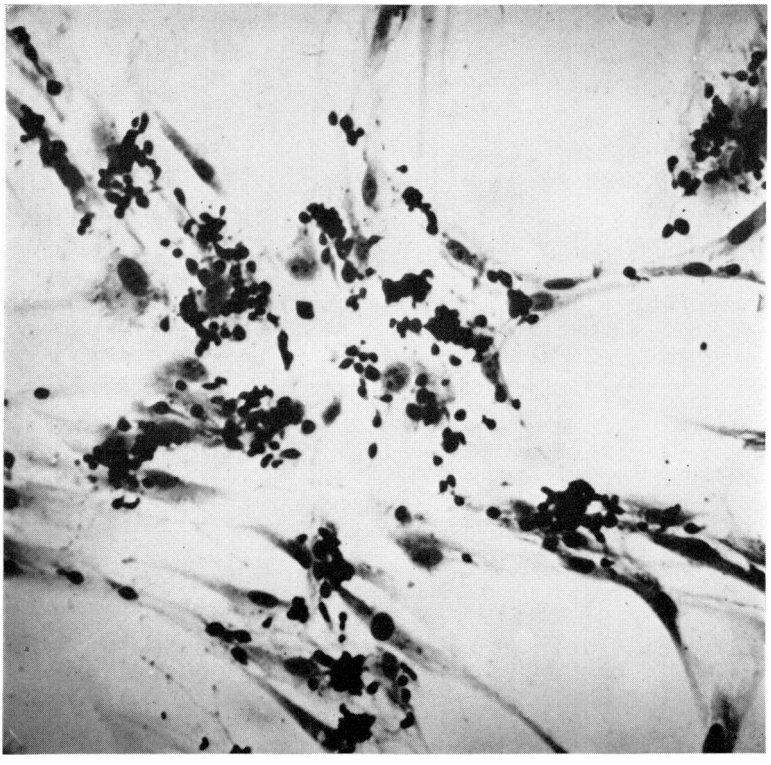

FIG. 6. Lymphocyte-target cell aggregation 3 days after addition of IgG (derived from a late anti-lymphocyte serum, see Fig. 1) in a concentration of 1:800 to the culture medium.

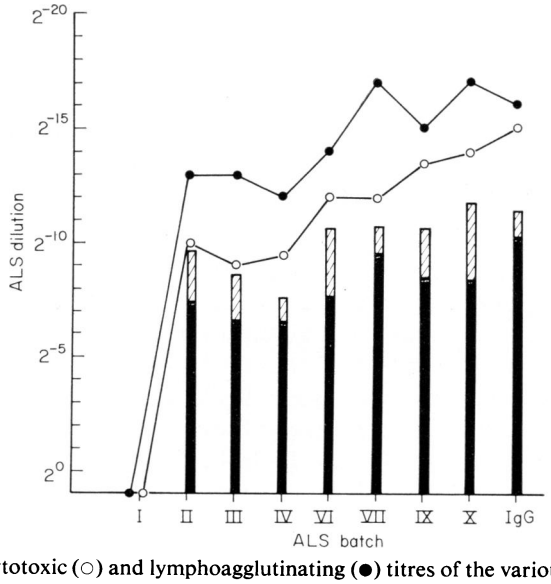

FIG. 7. Lymphocytotoxic (○) and lymphoagglutinating (●) titres of the various ALS batches in relation to their capacity to cause a partial (broken columns) and total (solid columns) inhibition, respectively, of PHA-induced lymphocyte cytotoxicity on sheep fibroblasts.

the inhibition of PHA-induced cytotoxic activity of lymphocytes on one hand and the lympho-agglutinating titres and the cytotoxic titres on the other hand. Sheep fibroblasts were used as targets to avoid the influence of ALS itself to induce lymphocyte-mediated cytotoxicity.

Interaction between different non-specific stimulators

As mentioned above lymphocytes pre-treated with PHA for 3 days in tissue culture tubes and carefully washed prior to their application to human fibroblast target cells were

TABLE 4. Cytotoxicity of human lymphocytes pre-treated with PHA, ALS and streptolysin O, respectively, on human fibroblasts (effect of PHA, ALS and streptolysin O added to the culture medium on cytotoxicity caused by 10^6 pre-treated lymphocytes)

Pre-treatment between day −3 and day 0 with:	Substances added to the culture medium on day 0			Plaque-formation* at day		
	PHA	ALS	Streptolysin O	+2	+4	+6
PHA 1:100				3·0 (2)	3·0 (3)	3·7 (3)
	1:100			3·3 (2)	3·3 (2)	3·3 (2)
		IV 1:50		1·0 (2)	0·7 (3)	0·7 (3)
			1:50	3·5 (2)	3·0 (2)	3·5 (2)
ALS IV 1:10				2 (1)	1·5 (2)	2·7 (3)
	1:100			3 (1)	3·0 (2)	3·3 (3)
		IV 1:50		0 (1)	0·5 (2)	0·7 (3)
			1:50		2 (1)	3 (1)
ALS VI 1:10						2·7 (3)
		VI 1:10				0·3 (3)
Streptolysin O 1:10 to 1:50				0·9 (8)	1·4 (8)	1·1 (8)
	1:100			3·3 (7)	3·4 (7)	3·4 (7)
		IV 1:50		1·2 (8)	1·4 (8)	1·6 (8)
			1:50	1·1 (8)	1·4 (8)	1·4 (8)

* Plaque-formation was graded between 0 and 4+ and expressed as the mean value of the experiments performed. Number of experiments within parentheses.

capable of causing plaques in the monolayer. This cytotoxic effect could be inhibited by ALS added to the culture medium. If the lymphocytes were pre-treated with ALS for 3 days a similar but usually weaker cytotoxic effect was obtained on human targets. This effect was potentiated if the culture medium was supplemented with PHA, but markedly reduced if ALS was added to the medium instead of PHA (Table 4). A cytotoxic capacity could also be induced in the lymphocytes by pre-treating them with streptolysin O (Table 4). When streptolysin was present in the culture medium the cytotoxic effect caused by PHA- or ALS-pre-treated lymphocytes was potentiated (Table 4). The cytotoxic effect caused by lymphocytes pre-treated with streptolysin O in a concentration of 1:10 for 3 days was further increased by addition of PHA to the Petri dish. An ALS (batch IV 1:50) which caused a pronounced inhibition of the cytotoxic effect exerted by PHA- or ALS-pre-treated lymphocytes was totally incapable of suppressing the cytotoxic effect caused by streptolysin-pre-treated lymphocytes (Table 4). Thus, ALS was a powerful inhibitor of plaque-formation

TABLE 5. Interaction between PHA, ALS and streptolysin O on lymphocyte-mediated cytotoxicity (10^6 lymphocytes were added to human fibroblast monolayers)

Substance in medium			Plaque-formation at day		
PHA	ALS IV	Streptolysin O	2	4	6
			0	0	0
1:100			++	+++	+++
	1:50		0	0	+
		1:50	0	0	0
1:100	1:50		0	+	++
1:100		1:50	++	++++	++++
1:100	1:50	1:50	0	+	++

caused by PHA- or ALS-pre-treated lymphocytes but it failed to suppress a similar cytotoxic effect induced by streptolysin. Various possible mechanisms for this discrepancy was investigated. It seemed possible that streptolysin-pre-treated lymphocytes could not react with ALS. However, it was found that streptolysin-treated cells were killed by ALS in the presence of rabbit complement, demonstrating that the antibodies could interact with the

TABLE 6. Inhibiting effect of ALS VII on plaque-formation caused by 10^6 human lymphocytes pre-treated with streptolysin O for 3 days at a concentration of 1:10 (streptolysin O 1:10 was also added to the culture medium of human and sheep fibroblasts, respectively)

Dose of ALS VII	Target cells	Plaque-formation at day	
		3	6
	Human	+++	+++
1:50	Human	+	+
1:100	Human	+	++
1:200	Human	+++	+++
1:400	Human	+++	+++
	Sheep	++++	++++
1:50	Sheep	0	0
1:100	Sheep	0	0
1:200	Sheep	+	+
1:400	Sheep	+	+
1:800	Sheep	+	+

cell surface. Horse γ-globulin was also demonstrated on lymphocytes pre-treated with streptolysin by the addition of fluoroscein-labelled goat antibodies against horse γ-globulin. Thus, the inefficiency of ALS to prevent plaque-formation caused by streptolysin-pre-treated lymphocytes was not due to a repulsion of the antibodies by the lymphocytes. Another possibility would be that streptolysin inactivated ALS. This was tested by adding streptolysin to cultures containing PHA and/or ALS. Since ALS suppressed plaque-formation induced by

PHA, it was tested whether streptolysin could abolish this suppression, as would be expected if streptolysin inactivated ALS. As can be seen in Table 5 this was not the case. Using a more potent ALS (VII) inhibition of plaque-formation caused by streptolysin-pre-treated lymphocytes could be obtained. This effect was more pronounced on sheep fibroblasts as compared to human (Table 6). However, the degree of inhibition was considerably smaller than that observed with the same ALS on the cytotoxic activity of PHA- or ALS-pre-treated lymphocytes.

DISCUSSION

Heterologous anti-lymphocyte serum in certain concentrations was found to induce a cytotoxic potential in normal human peripheral blood lymphocytes against allogeneic fibroblasts. Purification of the crude horse serum showed that this and all other properties of ALS discussed below were present in the 7S γ-globulin fraction of the serum. The finding that the destruction of the fibroblast monolayer was confined to the area of lymphocyte application makes it unlikely that the mechanism of the ALS-induced cytotoxicity should be a direct toxic effect of the antiserum against the target cells. However, a local enrichment of ALS would be expected at the area of lymphocyte application, since the antigenic receptors on the lymphocytes would concentrate ALS to the lymphocyte surfaces. The finding that heating of the lymphocytes to 48·5°C for 30 min totally abolished their capacity to kill fibroblasts in the presence of ALS makes such an explanation unlikely, since that temperature is too low to destroy the cell antigens and, therefore, should not interfere with the binding of ALS to the lymphocytes. These findings re-emphasize the importance of living and metabolically active lymphocytes for expression of cytotoxicity in the presence of ALS.

ALS could not render the lymphocytes cytotoxic against sheep fibroblasts. In this respect ALS differed from the other non-specific stimulators used, since lymphocytes in the presence of PHA and streptolysin O as well were fully capable of destroying sheep fibroblasts (Tables 3 and 6). A possible explanation for this difference would be that the ALS-antibody molecules have to create a close contact between the lymphocytes and the target cells for cytotoxicity to occur. Since the antibody is bivalent and directed against human tissues, such a close aggregation could be established between different cells of human origin but not between human lymphocytes and sheep fibroblasts. Therefore, the ALS-stimulated lymphocytes possessing a cytotoxic potential would not be able to express this on sheep fibroblasts, since the necessary intimate contact would not be formed. Actually, microscopical investigations showed that no lymphocyte aggregation was obtained on sheep fibroblasts in the presence of ALS. According to this concept, a very close contact must be established between the triggered lymphocytes and target cells, since one layer of antibody molecules on the lymphocytes was sufficient to prevent cytotoxicity.

On human fibroblasts a lymphocyte–fibroblast aggregation could be demonstrated (Table 1). This aggregation was maximal with the ALS-concentrations which induced a maximal cytotoxic activity of the lymphocytes. The experiments suggest, therefore, that ALS acts in two steps in the cytotoxic process: (1) it stimulates the lymphocytes to aquire a cytotoxic potential; and (2) it creates a close contact between the cytotoxic lymphocyte and the target cell. ALS may stimulate the lymphocytes into a cytotoxic state by the same mechanism as PHA, streptolysin O and other non-specific stimulators, but the actual contact between the lymphocytes and the target cells may differ with the various substances.

ALS induced a cytotoxic activity of the lymphocytes only in a limited concentration range, above and below which no cytotoxicity was obtained. The finding that increasing dilutions of the antiserum resulted in decreasing cytotoxicity was probably due to a diminishing capacity both to stimulate the lymphocyte into a cytotoxic state and to establish close contact between the effector and the target cells. Fig. 2 and Table 1 show that the capacity of ALS to induce lymphocyte-mediated cytotoxicity declines in parallel with its ability to stimulate lymphocyte DNA-synthesis and its potency to cause lymphocyte–target aggregation. Although it has been shown previously (Lundgren & Möller, 1969) that there was a parallelism between stimulation of DNA-synthesis and induction of cytotoxicity, the two processes were not connected, since the DNA-synthesis of the lymphocytes could be suppressed completely without affecting their cytotoxic capacity. The inability of ALS in higher concentrations to induce a cytotoxic activity of the lymphocytes must probably be explained in other terms. Although ALS was shown to be toxic to the lymphocytes (Table 1) it is unlikely that this could explain the results, since no obvious differences in toxicity could be shown between the various concentrations of ALS. It may be speculated that ALS by virtue of its bivalent structure can create a close connection between the lymphocyte and the target cell, which would be a necessary pre-requisite for cytotoxicity to occur and, consequently, that an excess of ALS in the culture medium would block the antigenic determinants on the lymphocytes and the target cells as well making the bridging between lymphocyte and target cell more difficult. This hypothesis predicts that an optimal degree of cytotoxicity would be obtained by pretreating the lymphocytes with ALS in a separate vessel. By this procedure the lymphocytes would aquire a cytotoxic potential and be coated with antibodies possessing free sites for attachment to antigenic determinants on the fibroblasts when transferred to monolayer cultures. Direct experiments confirmed that pre-treatment of lymphocytes with ALS caused a stronger cytotoxicity than if ALS was continuously present in the culture medium. Other or co-operating mechanisms for the inefficiency of ALS in high concentrations to render lymphocytes cytotoxic would be the tendency of ALS in these concentrations to aggregate the lymphocytes to each other in large aggregates as demonstrated in Fig. 5, thereby reducing the effective number of lymphocyte–target cell interactions and/or the inferior capacity to stimulate the DNA-synthesis of the lymphocytes (Fig. 2) possibly reflecting a lower stimulation of their cytotoxic capacity.

ALS was demonstrated to suppress the well-documented effect of PHA to induce a cytotoxic capacity in human lymphocytes against various target cells (Holm, Perlmann & Werner, 1964; Möller *et al.*, 1966; Lundgren & Möller, 1969). A similar inhibition of lymphocyte-mediated cytotoxicity on Chang cells induced by PHA was shown by Holm & Perlmann (1969) although they could not demonstrate any cytotoxicity-inducing effect by ALS itself. Evidence cited above suggest that this inhibition is not due to an action of ALS on the fibroblasts or on PHA, but due to an action on the lymphocytes. There are many possible mechanisms for such an effect of ALS (Levey & Medawar, 1966, 1967a, b). Complement-dependent lysis of the lymphocytes was excluded, since no heat-labile complement factors were present in the system. A direct toxic effect of ALS on the lymphocytes was also considered unlikely as an explanation, since concentrations of ALS sufficient to abolish the cytotoxic effect totally on sheep fibroblasts were inefficient to do so on human fibroblasts. Furthermore, ALS II and IV which were shown to be most toxic to the lymphocytes (Table 1) were least effective in preventing PHA-induced plaque-formation (Fig. 7). A 'blind-folding' mechanism of the lymphocytes, implying that ALS blocks receptors on the lymphocyte

necessary for recognition of foreign antigens and induction of an immune response, can also be excluded as the whole explanation in this system, since lymphocytes pre-treated with stimulating substances (PHA, ALS or streptolysin O), and which, therefore, were already transformed to cytotoxically active cells, were inhibited by ALS. The same findings make the concept of 'sterile activation' unlikely as the mechanism by which the suppression of lymphocyte-mediated cytotoxicity occurs in this system, since this concept implies an action on the induction phase. The experiments agree with the possibility that ALS acts directly on the effector mechanism of the cytotoxic reaction, i.e. by coating the lymphocytes, making them incapable of making close contact to the target cells. However, ALS has previously been reported (Mosedale, Felstead & Parke, 1968; Lundgren, Collste & Möller, 1968a) to inhibit the PHA-induced stimulation of lymphocyte DNA-synthesis. The mechanism for this inhibition is unknown but it is highly unlikely that it should be due to a coating of the lymphocytes by ALS.

From the above discussion concerning the mechanism for the lymphocyte-mediated cytotoxicity induced by ALS it follows that it is of importance that the ALS-molecules create connections between the human lymphocytes and the human target cells. When sheep fibroblasts were used as targets no contact would be expected to be established and the experiments also showed that a cytotoxic effect could not be obtained. The PHA-stimulated lymphocytes would be coated by ALS in the same way. It seems probable that this layer of ALS, lacking ability to create contact with the sheep tissue, prevents the PHA-mediated aggregation of the lymphocytes to the sheep fibroblasts, which is a necessary prerequisite for cytotoxicity to occur (Lundgren & Möller, 1969). The experiments in Table 3 showing that pre-treatment of lymphocytes with ALS for 3 days markedly suppressed the cytotoxic reaction on sheep fibroblasts in the presence of PHA also supports such an hypothesis. Using human fibroblasts the antibody-connection between lymphocyte and target cell should be established in the range of ALS-concentrations capable to induce cytotoxicity (Table 1). Excess of antibodies would destroy this connection. Since PHA-stimulated lymphocytes would be be coated by ALS in the same way, a similar excess of antibodies would also interfere with PHA-aggregation. Fig. 3 shows that a complete suppression of cytotoxicity was obtained with these doses of ALS. By comparing Table 1 and Fig. 3 it can be seen that the concentration range of ALS, which by itself induced cytotoxicity, was exactly the same as that causing partial suppression of PHA-induced cytotoxicity. This suggests that the layer of antibody molecules bridging the effector and target cells separates the cells to such an extent that the close aggregation necessary for the strong cytotoxicity obtained in the presence of PHA is prevented. At higher dilutions of ALS, where this separation cannot be completely established, no suppression of the cytotoxic effect was obtained. It is also of interest that the concentrations of ALS causing partial suppression of PHA-induced lymphocyte-mediated cytotoxicity on human fibroblasts (Fig. 3) coincides with those completely suppressing the same cytotoxicity on sheep fibroblasts. This further strengthens the hypothesis, since a concentration of antibody on the lymphocytes of sufficient density to establish a close contact with human fibroblasts would also be expected to interfere with the PHA-aggregation of the lymphocytes to sheep fibroblasts.

The suppressing effect of ALS on the cytotoxic activity of streptolysin-treated lymphocytes was less efficient than the inhibiting effect on cytotoxicity caused by lymphocytes treated with PHA or ALS (Table 4), which may indicate that streptolysin activates the lymphocytes in a special way. One possible explanation would be that streptolysin-activated lymphocytes

do not need a very close contact with the target cells to kill them. That the killing should be effectuated by a release of a toxic substance from the lymphocytes is unlikely, however, since streptolysin-activated lymphocytes could not kill fibroblast target cells which were covered by a very thin layer of agar (Lundgren, unpublished data).

In conclusion the experiments presented suggest that ALS suppresses the cytotoxic activity of PHA-, ALS- and streptolysin-treated lymphocytes by coating the lymphocytes with antibodies. It is possible that a similar mechanism may operate *in vivo* where it would be indistinguishable from a 'blind-folding' effect and could be a partial explanation of the *in vivo* immunosuppressive effect. Investigations by Brent, Courtenay & Gowland (1967) support this hypothesis. They showed that pre-treatment of lymphocytes by ALS abolished their capacity to induce a graft-*versus*-host reaction in histoincompatible newborn mice. This capacity was recovered after trypsinization of the lymphocytes. Other data, however, indicate that the complement-binding sites of the antibody molecule are necessary for immunosuppression *in vivo* (Anderson, James & Woodruff, 1968) and that the immunosuppressive effect of ALS will last over several cell divisons (Levey & Medawar, 1967a), during which a heavy dilution of ALS molecules on the surface of the lymphocytes would be expected. ALS has many properties and it is likely that a combination of different mechanisms are responsible for the immunosuppressive effect *in vivo*. The present method may be one possibility to measure one of these mechanisms *in vitro*.

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REFERENCES

ANDERSON, N.F., JAMES, K. & WOODRUFF, M.F.A. (1968) Effect of acid treatment on the immunosuppressive properties of antilymphocytic IgG. *Nature (Lond.)*, **218**, 771.

BRENT, L., COURTENAY, T. & GOWLAND, G. (1967) Immunological reactivity of lymphoid cells after treatment with antilymphocytic serum. *Nature (Lond.)*, **215**, 1461.

GRÄSBECK, R., NORDMAN, C. & DE LA CHAPELLE, A. (1963) Mitogenic action of antileucocyte immune serum on peripheral leucocytes *in vitro*. *Lancet*, **ii**, 385.

HOLM, G., PERLMANN, P. & WERNER, B. (1964) Phytohaemagglutinin-induced cytotoxic action of normal lymphoid cells on cells in tissue culture. *Nature (Lond.)*, **203**, 841.

HOLM, G. & PERLMANN, P. (1969) Inhibition of cytotoxic lymphocytes by antilymphocytic serum. *Transplant. Proc.* **1**, 420.

LEVEY, R.H. & MEDAWAR, P.B. (1966) Nature and mode of action of antilymphocytic artiserum. *Proc. nat. Acad. Sci. (Wash.)*, **56**, 1130.

LEVEY, R.H. & MEDAWAR, P.B. (1967a) Further experiments on the action of antilymphocytic antiserum. *Proc. nat. Acad. Sci. (Wash.)*, **58**, 470.

LEVEY, R.H. & MEDAWAR, P.B. (1967b) The mode of action of antilymphocytic serum. *Antilymphocytic Serum: Ciba Foundation Study Group* 29 (Ed. by G.E.W. Wolstenholme and M. J. O'Connor). Churchill, London.

LUNDGREN, G., COLLSTE, L. & MÖLLER, G. (1968a) Cytotoxicity of human lymphocytes: antagonism between inducing processes. *Nature (Lond.)*, **220**, 289.

LUNDGREN, G., ZUKOSKI, Ch. F. & MÖLLER, G. (1968b) Differential effects of human granulocytes and lymphocytes on human fibroblasts *in vitro*. *Clin. exp. Immunol.* **3**, 817.

LUNDGREN, G. & MÖLLER, G. (1969) Non-specific induction of cytotoxicity in normal human lymphocytes *in vitro*: Studies of mechanism and specificity of the reaction. *Clin. exp. Immunol.* **4,** 435.

MONACO, A.P., WOOD, M.L., VAN DER WERF, B.A. & RUSSELL, P.S. (1967) Effects of antilymphocyte serum in mice, dogs, and man. *Antilymphocytic Serum. Ciba Foundation Study Group* 29 (Ed. by G. E. W. Wolstenholme and M. J. O'Connor), p. 111. Churchill, London.

MOSEDALE, B., FELSTEAD, K.J. & PARKE, J.A.C. (1968) Effect of anti-lymphocyte serum on the response of human and mouse lymphocytes to PHA. *Nature* (*Lond.*), **218,** 983.

MÖLLER, G., BECKMAN, V. & LUNDGREN, G. (1966) *In vitro* destruction of human fibroblasts by non-immune lymphoid cells. *Nature* (*Lond.*), **212,** 1203.

MÖLLER, G., LUNDGREN, G., ZUKOSKI, CH.F. & BECKMAN, V. (1967) Interaction between allogeneic cells of human origin: An *in vitro* model of graft rejection. *Scand. J. Urol. Nephrol.* **1,** 199.

PERPER, R.J., OKIMOTO, J.T., COCHRUM, K.C., RAMSEY, N. & NAJARIAN, J.S. (1967) A rapid method for purification of large quantities of anti-lymphocytic serum. *Proc. Soc. exp. Biol.* (*N.Y.*), **125,** 575.

REICH, E., FRANKLIN, R.M., SHATKIN, A.J. & TATUM, E.L. (1961) Effect of Actinomycin D on cellular nucleic acid synthesis and virus production. *Science,* **134,** 556.

STARZL, T.E., PORTER, K.A., IWASAKI, Y., MARCHIORO, T.L. & KASHIWAGI, N. (1967) The use of heterologous antilymphocyte globulin in human renal homotransplantation. *Antilymphocytic Serum: Ciba Foundation Study Group* 29 (Ed. by G. E. W. Wolstenholme and M. J. O'Connor), p. 4. Churchill, London.

STRANDER, H. & CANTELL, K. (1966) Production of interferon by human leukocytes *in vitro*. *Ann. Med. exp. Fenn.* **44,** 265.

TRAEGER, J., CARRAZ, M., FRIES, D., PERRIN, J., SAUBIER, E., BERNHARDT, J.P., REVILLARD, J.P., BONNET, P., ARCHIMBAUD, J.P. & BROCHIER, J. (1969) Studies of anti-lymphocyte globulins made from thoracid duct lymyhocytes. *Transplant. Proc.* **1,** 455.

WOODRUFF, M.F.A. & ANDERSON, N.F. (1963) Effect of lymphocyte depletion by thoracic duct fistula and administration of antilymphocytic serum on survival of skin homografts in rats. *Nature* (*Lond.*), **200,** 702.