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IN VITRO CYTOTOXIC EFFECTS OF LYMPHOID CELLS FROM RATS WITH EXPERIMENTAL AUTOIMMUNE NEPHROSIS

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SUMMARY

A nephrotic syndrome was induced in randomly bred Sprague-Dawley rats by repeated intraperitoneal injections of homologous kidney extract and Freund's complete adjuvant. Cell injury *in vitro* was measured by estimating the release of radioactivity from damaged target cells labelled with ^{14}C-thymidine. Various conditions influencing the reproducibility of the isotope method were investigated.

Blood lymphoid cells, lymph node cells and spleen cells from these rats damaged primary monolayer cultures of rat kidney cells after 18 hr incubation. Lymphoid cells from liver sensitized rats were also cytotoxic to rat kidney cells, whereas lymphoid cells from rats injected with other rat tissue extracts (lung or spleen) or given only adjuvant did not react. Primary cultures of rat lung cells were sensitive while cells of a rat liver cell strain were resistant to the cytotoxic action of lymphoid cells from kidney sensitized rats. Sera from nephrotic rats did not damage rat kidney cells under the experimental conditions used here.

The mechanisms of tissue damage in human and experimental autoimmune diseases are largely unknown. Data accumulated from histological observations of the tissue lesions suggest that lymphoid cells are involved in the development of such diseases (Waksman, 1962). The successful passive transfer of allergic encephalitis in rats (Paterson, 1960) and of autoimmune nephrosis in rats (Hess, Ashworth & Ziff, 1962; Heymann *et al.*, 1962) by lymphoid cells from diseased animals, further supports the hypothesis that 'sensitized' lymphoid cells carry some factors necessary for initiating the tissue lesions.

In investigations of the ability of lymphocytes from patients with diseases associated with autoimmunity to cause tissue damage, tissue culture methods are of great value. Using these techniques, destruction of the specific target cells has been demonstrated with lymphoid cells from rats having experimental autoimmune thyroiditis (Biörklund, 1964) and experimental allergic encephalitis (Koprowski & Fernandes, 1962; Berg & Källén, 1963), and with lymphoid cells from patients having ulcerative colitis (Perlmann & Broberger, 1963) and multiple sclerosis (Berg & Källén, 1964).

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This article describes the cytotoxic effect of lymphoid cells from rats with experimental autoimmune nephrosis, on rat kidney cells in tissue culture.

MATERIALS AND METHODS

Rats

Female albino Sprague-Dawley rats, randomly bred, were used throughout. The rats were fed rat cake and tap water daily. They weighed 150–200 g at the beginning of the experiments.

Immunization

The method described by Heymann *et al.* (1959) was essentially followed. Kidneys from normal female rats were perfused *in situ* with cold isotonic phosphate buffer (pH 7·2). They were then removed and homogenized in an equal volume of saline buffer. The homogenate was centrifuged at 3000 rev/min for 1 hr at 4°C. The supernatant was poured off and stored in small portions at —20°C. Antigen extracts were prepared in the same way from rat liver, lung and spleen tissue. The protein content of 0·1 ml of the tissue extract, determined according to Lowry *et al.* (1951), was: 2·6–3·5 mg for kidney, 2·9–3·4 mg for liver, 1·3–1·5 mg for lung, and 2·9 mg for spleen.

One group of rats was injected intraperitoneally every second week with an emulsion consisting of 0·4 ml kidney homogenate diluted 1 : 4 with buffer, and 0·4 ml complete Freund's adjuvant, (85 ml paraffin oil, 15 ml Arlacel A and 200 mg *Mycobacterium tuberculosis*, bovine type). Three groups of animals were immunized in the same way with rat liver-, lung- or spleen-extracts. A fifth group was injected with Freund's adjuvant alone.

Urine protein determination

Urine was collected during a 24 hr period every second week. Water was given to the animals during the period of collection. Thymol was added to the sampling vessel as a preservative. Protein was determined as described by Shevky & Stafford (1923). The protein content in the urine of normal Sprague-Dawley rats did not exceed 40 mg/day.

Tissue cultures

Kidneys were removed from 2–7-day-old (female) rats and trimmed free of fat and connective tissue. They were then minced into small pieces and washed twice. The tissue fragments were treated with 0·33% trypsin in phosphate buffer, pH 7·6, at 37°C for 30 min with continuous stirring. After sedimentation of cell clumps and debris, the cell suspension was poured off, diluted with one volume of cold medium and centrifuged at 800 rev/min for 10 min. After washing, the cells were suspended in Eagle's medium (supplemented with 100 units of penicillin and 100 μg of streptomycin per ml) containing 15% heat-inactivated calf serum. The cells were counted in a Bürker chamber after staining of dead cells with lissamine green (Holmberg, 1961). Five million living cells suspended in 30 ml Eagle's medium, were pipetted into Petri dishes containing fifteen to twenty coverglasses (22 × 22 mm). The cultures were incubated at 37°C in an atmosphere of air with 5% carbon dioxide. After 1–2 days the medium was changed and 2–3 μc of thymidine-2-^{14}C (specific activity 26–36 mc/m-mole; Radiochemical Centre, Amersham, England) in 30 ml medium

was added to some of the cultures. The cultures were then incubated for an additional 2–4 days.

Primary cultures of lung cells from newborn rats were prepared in the same way. Liver and kidney cell strains from Sprague-Dawley rats were grown in suspension cultures. From these stocks, sub-cultures were started in Petri dishes with coverslips and prepared as described above. The liver strain consisted of parenchymal rat liver cells cloned from a primary culture of adult rat liver. It had been maintained *in vitro* for about 4 months when used for the cytotoxicity experiments. The kidney strain was of the same age and consisted exclusively of parenchymal cells derived from a primary adult rat kidney culture by cloning. In the electron microscope these cells showed the typical appearance of parenchymal kidney cells, with a well-developed brush border.

Preparation of lymphoid cells

Rat blood was collected by heart puncture under sterile conditions and heparinized (Heparin, Vitrum; preservative-free). After sedimentation for 1 hr at room temperature, the tubes were centrifuged for 10 min at 400 rev/min. Buffy coat was removed and centrifuged at 1500 rev/min for 5 min. The cells were then treated with 0·35% sodium chloride for 1 min in order to lyse the remaining red cells (Janowsky, Rosenau & Moon, 1964). After centrifugation, the cells were washed twice in Eagle's medium containing 5% heat-inactivated calf serum. Cell viability was determined after addition of an equal volume of 0·1% trypan blue in Hanks's solution.

Lymph nodes were dissected out aseptically from cervical, axillary, mesenteric and retroperitoneal areas, freed from fat and connective tissue and cut into small pieces. The spleens were treated in the same way after perfusion with Hanks's solution. The pieces were then placed on metal grids as described below. In some experiments suspension of spleen or lymph node cells were prepared by mincing the tissue in medium with 5% calf serum and filtering it through nylon gauze. The cells were then washed twice and the number of viable cells determined as described.

Sera

Sera were taken from all rats by heart puncture on the same occasion as the lymphoid cells. All sera were inactivated at 56°C for 30 min and stored in small portions at —20°C.

Determination of cytotoxicity

The primary cultures were used after outgrowth for 4–8 days. At that time, the kidney cultures consisted of a partially merging layer of parenchymal cells and fibroblasts, with a predominance of the epithelial cell type.

1. *Isotope release.* A schematic outline of the technique used (Vainio *et al.*, 1964) is seen in Fig. 1. The cell cultures were washed four times with medium in order to remove adsorbed isotope. The coverslips were then transferred to small Petri dishes (50 mm diameter) with one glass in each dish. A stainless steel mesh platform with 20 × 20 mm surface area on 5 mm high supports (Trowell, 1959) was placed over the coverslip. The platform was covered with lens paper and an equal number of lymph node or spleen fragments was put on each grid. The Petri dish was filled up with medium to the level of the lens paper. In some experiments

in which there were suspensions of lymphoid cells the grids were omitted. The dishes were incubated at 37°C in a humid atmosphere of air with 5% carbon dioxide. During incubation lymphoid cells fell through the mesh onto the tissue culture cells.

After incubation, grids and tissue pieces were removed. The medium was pipetted off and centrifuged for 5 min at 1500 rev/min. The cell-free supernatant (Sup. I) was withdrawn and cooled. The cell sediment (Cell sed. I) as well as the cells, which remained in the dishes, were both treated with 0·25% trypsin (phosphate buffer, pH 7·6) for 30 min at room temperature with occasional shaking. Both cell suspensions were combined in centrifuge tubes and cooled. In order to remove all cells from the dishes, the latter were washed twice with cold Hanks's solution. The cells were then spun down at 2000 rev/min for 6 min and the supernatant (Sup. II) was separated from the trypsin treated cells (Cell sed. II).

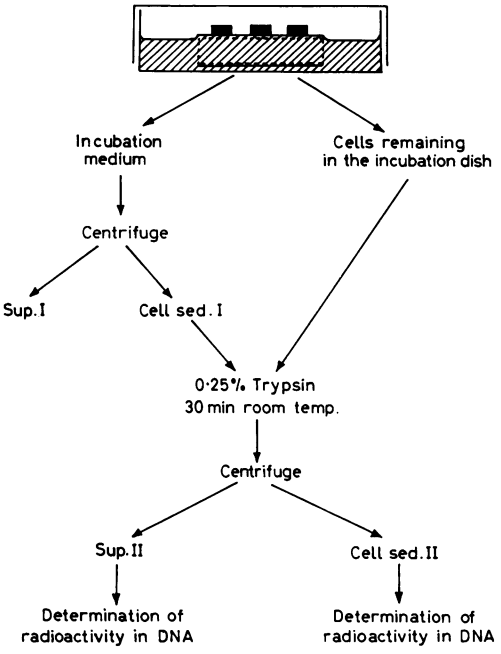

FIG. 1. Diagram of the method used for determining the cytotoxic action of lymphoid cells on cells in tissue culture.

$$\text{Cell damage} = \frac{\text{Counts/min in Sup. II} \times 100}{\text{Counts/min in Sup. II} + \text{Cell sed. II}}$$

Nucleic acids were extracted from Sup. II and Cell sed. II with a trichloroacetic acid (TCA) procedure (Volkin & Cohn, 1959). Nucleic acids and proteins were precipitated by addition of an equal volume of cold 10% TCA. The precipitate was washed once in cold 5% TCA and then extracted twice with 5% TCA at 90°C. TCA was extracted from the nucleic acid solution with several changes of ether. This nucleic acid extract contained both desoxyribonucleic acid (DNA) and ribonucleic acid (RNA). Since ^{14}C-thymidine is a specific precursor of DNA, the presence of RNA could be disregarded and the extract will be called 'DNA' in the text and tables following. The DNA extracts (and in some experiments

the TCA soluble 'nucleotides' and the TCA precipitates) were plated and dried on plastic planchettes and counted at infinite thinness in a thin-windowed gas-flow counter at 10% efficiency. The DNA-bound isotope, released after trypsin treatment of the cells, was expressed as a percentage of the activity in DNA of cells + trypsin supernatant, and was used as a measure of cell damage. All percentage figures in Tables 4–7 are expressed in this way. The radioactivity (counts/min) in the same tables is the amount of isotope in DNA of Sup. II + Cell sed. II.

2. *Microscopical observations.* Similar experiments were set up with unlabelled cell cultures. After incubation, the coverglasses were washed once in serum-free medium, fixed in Carnoy's fixative or in absolute methanol and stained with May-Grünwald and Giemsa stain. The cultures were observed for aggregation of lymphoid cells to the target cells and for degenerative alterations in the latter.

Haemagglutination

All rat sera to be tested were absorbed with an equal volume of a 50% sheep red blood cell suspension, in order to remove heterophilic antibodies. A rat kidney homogenate, prepared as described above, centrifuged at 10 000 rev/min for 1 hr at 4°C, and diluted with phosphate buffer (pH 6·4) to give a protein concentration of 3·0 mg/ml, was used for sensitizing the cells.

The tanned cell haemagglutination method as described by Stavitsky (1954) was followed. The titrations were performed in Perspex agglutination trays. Serial dilutions were prepared from the rat serum. To 0·1 ml of each serum dilution was added 0·1 ml of a 0·5% suspension of sensitized sheep red cells. Controls with non-sensitized, tannic acid treated cells were always set up. Positive controls with rabbit anti-rat kidney serum were also included. The tests were read after 4 and 18 hr incubation at room temperature.

Immunodiffusion

The method described by Ouchterlony (1958) was used. One per cent special Difco agar Noble was prepared in phosphate buffer, pH 7·4. Two rat kidney extracts were used. One was an aliquot of that used for the haemagglutination test. The other was extracted from rat kidney after homogenization with sodium desoxycholate and lubrol W (Morgan, Perlmann & Hultin, 1961). Normal rat sera were included in every test. Five fillings of antigen and antisera were made during the first 48 hr. The plates were incubated at 37°C for 4 days and then at 4°C for an additional 14 days.

RESULTS

Incidence of Nephrosis

The presence of increased amounts of protein in the urine was taken as an indication of kidney disease. Some of the rats immunized with kidney antigens already developed a nephrotic syndrome after three injections. After seven injections 75% of the rats in this group were nephrotic (Table 1). Further immunization did not increase the incidence of nephrosis. A significant number of animals in the groups injected with liver homogenate also showed an abnormal excretion of protein in the urine. Occasionally a slight proteinuria was seen in rats immunized with lung antigens or with Freund's adjuvant alone.

One or two days after the initial intraperitoneal injections rats in all groups had transient ascites due to an aseptic peritonitis. With increasing number of injections the peritoneal cavity became occluded by fibrous adherences. At this stage most of the nephrotic rats had a constant ascites of between 10 and 150 ml. Only minute amounts of ascites fluid were present in healthy control rats. During the course of the disease the nephrotic rats lost weight and became cachectic. Manifest peripheral oedema was not observed. Improvement did not occur during the 4-month period of observation.

TABLE 1. Incidence of nephrosis*

Protein in urine (mg/day)	No. of rats immunized with				
	Kidney	Liver	Lung	Spleen	Adjuvant
<40	11	11	14	10	39
40–80	16	3	1	0	5
>80	16	1	0	0	0
Incidence of nephrosis (%)	74	27	7	0	11

*The nephrotic rats had been given three to seven injections. The non-reactors were injected six times or more.

The Cytotoxic Action of Lymphoid Cells

Microscopical observations

In the experiments with primary rat kidney cultures incubated with lymphoid cells from nephrotic rats, the most apparent microscopical finding was an aggregation of lymphocytes to the target cells. This phenomenon was already obvious after 3 hr, when no kidney cell damage could be detected under the microscope or with the isotope technique. With longer time of incubation, this clustering became more pronounced. Thus after 18 hr, colonies of epithelial kidney cells were often covered by a white cell mixture in which lymphocytes predominated. The same aggregation was also found between lymphoid cells and fibroblast-like cells in the culture. Several kidney cells in contact with lymphoid cells then showed vacuolization of the cytoplasm and pyknotic nuclei. After 48 hr the degenerative alterations of the kidney cells were more pronounced.

In the experiments with lymphoid cells from rats immunized with liver homogenate, these cells became attached to the kidney cells to the same extent as did lymphoid cells from kidney sensitized rats. Lymphoid cells from animals immunized with lung tissue showed a slight tendency to aggregate in some experiments. However, aggregation was clearly less than in the two other groups. Lymphocytes from spleen sensitized or adjuvant sensitized rats showed no, or very faint, clustering.

Isotope experiments

Evaluation of the isotope method. For accurate measurements of isotope release as an indicator of cell injury, it was necessary to extract DNA. The proportion of the total isotope

recovered in the DNA-extracts and the isotope-distribution in the different fractions obtained during processing of the cells (Fig. 1) were investigated in the following way. Rat kidney cells in suspension culture were labelled with ^{14}C-thymidine. After washing, cell samples containing 10^5 living cells were transferred to tissue culture tubes with 1·5 ml Eagle's medium and 5% calf serum. The fraction of dead cells in these samples at the beginning of the experiment was 21%. Five tubes were incubated for 24 hr and another five for 48 hr. The tubes were then processed as shown in Fig. 1 (cf. Materials and Methods). Sup. I, Sup. II and Cell sed. II were each extracted separately with TCA as described above. The isotope was determined in the washes with cold TCA ('nucleotides'), in the hot TCA extracts ('DNA') and in the remaining precipitate ('residue').

TABLE 2. The influence of time of incubation on the distribution of isotope released from rat kidney cells labelled with ^{14}C-thymidine*

Time of incubation (hours)	Fractions	Radioactivity (counts/min)			Radioactivity (% release)†		
		Sup. I	Sup. II	Cell sed. II	Sup. I	Sup. I + II	Sup. II
24	Nucleotides	10 ± 5	15 ± 10	40 ± 18			
	DNA	41 ± 3	226 ± 21	680 ± 26	4·5 ± 0·7	28·2 ± 1·7	24·9 ± 2·0
	Residue	5 ± 1	26 ± 4	44 ± 7			
	Sum	56 ± 6	267 ± 25	764 ± 39	5·2 ± 0·6	29·7 ± 1·8	25·9 ± 2·3
48	Nucleotides	30 ± 6	16 ± 7	43 ± 12			
	DNA	86 ± 13	306 ± 36	538 ± 80	9·3 ± 1·8	42·2 ± 2·6	36·3 ± 2·5
	Residue	13 ± 2	32 ± 3	32 ± 4			
	Sum	129 ± 17	354 ± 34	613 ± 60	11·9 ± 1·8	43·9 ± 2·3	36·3 ± 2·4

*Tissue culture tubes, containing 10^5 kidney cells in 1·5 ml Eagle's medium with 5% heat-inactivated calf serum were incubated for 24 or 48 hr. Each tube was treated as described in the text and Fig. 1. All values in this table are means ± SD calculated from five independent incubations.

†Computed from isotope in the DNA-extracts or from the sum of radioactivities recovered from three different fractions.

$$\text{Per cent release to Sup. I} = \frac{(\text{Sup. I}) \times 100}{\text{Sup. I} + \text{Sup. II} + \text{Cell sed. II}}$$

$$\text{Per cent release to Sup. I + II} = \frac{(\text{Sup. I + II}) \times 100}{\text{Sup. I} + \text{Sup. II} + \text{Cell sed. II}}$$

$$\text{Per cent release to Sup. II} = \frac{(\text{Sup. II}) \times 100}{\text{Sup. II} + \text{Cell sed. II}}$$

Table 2 gives the results of this experiment. Each figure in this table represents mean value and standard deviation, calculated from five independent incubations. The percentages of isotope release, based either on the isotope in DNA or on the total isotope recovered, are also given in the table. As can be seen, the bulk of isotope both in Sup. II and in the Cell sed. II was found in DNA. Therefore the percentage of isotope released to Sup. II, as based on DNA-bound radioactivity, was very close to that calculated from the total

radioactivity recovered in Sup. II and Cell sed. II. Table 2 also shows that the isotope release into the incubation medium (Sup. I) after 24 hr was low and the release into Sup. II could therefore be used as a simple measure of cell damage. At 48 hr a little more isotope was found in Sup. I and the nucleotide fraction of this supernatant was comparatively larger, possibly indicating a slight breakdown of DNA during the course of the incubation. However, because of the small amounts of isotope in Sup. I, this loss was negligible.

In the experiment shown in Table 2 lymphoid cells were not present. The influence of such cells on the isotope release into Sup. I was studied in some experiments in which rat kidney cells and rat lymphocytes were incubated together. Table 3 gives the data from two typical experiments. Primary rat kidney cultures, labelled with ^{14}C-thymidine, were incubated for 18 hr with pieces of rat spleen on metal grids. DNA was extracted from Sup. I,

TABLE 3. The effect of lymphocytes on the release of isotope to the incubation medium. Rat kidney cells incubated with rat spleen cells* for 18 hr

Experiment No.	Spleen cells from	Radioactivity		
		Counts/min†	Per cent release Sup I + II‡	Sup. II§
1	Rat immunized with kidney	443	70·2	67·8
	Rat immunized with adjuvant	655	53·5	52·3
	Normal rat	411	49·6	40·7
	No spleen cells	234	19·3	15·6
2	Rat immunized with kidney	394	32·5	32·0
	Rat immunized with adjuvant	221	20·9	20·0
	Normal rat	594	17·8	16·2
	No spleen cells	306	17·2	17·2

*Pieces of spleen tissue incubated on metal grids.

†Isotope in DNA in Sup. I + Sup. II + Cell sed. II.

‡Per cent release to Sup. I + II = $\frac{(\text{Sup. I + Sup. II}) \times 100}{\text{Sup. I + Sup. II + Cell sed. II}}$

§Per cent release to Sup. II = $\frac{(\text{Sup. II}) \times 100}{\text{Sup. II + Cell sed. II}}$

Sup. II and Cell sed. II. In spite of the pronounced cell damage caused by the addition of lymphoid cells in Experiment 1, the isotope recovered from the incubation medium was small. In Experiment 2, less than 10 counts/min were extracted as DNA from Sup. I. In similar experiments the total isotope was measured directly without preceding extraction of DNA. Again, only relatively small amounts of isotope were found in the incubation medium (Sup. I), even if cell damage was considerable. This indicates that the fraction of isotope-labelled acid-soluble nucleotides in Sup. I was small even in the presence of lymphocytes.

The cytotoxic actions of lymphoid cells. All rats were given a booster injection before the experiments. Cytotoxic effects of lymphoid cells on rat kidney cells were not observed when the experiment was performed earlier than 8 days or later than 11 days after this booster

TABLE 4. Cytotoxic effect of spleen cells from nephrotic rats on primary cultures of rat kidney cells*

Method of incubation	Experiment No.	Rats immunized with kidney		Rats immunized with adjuvant		Normal rats		No spleen cells	
		Radioactivity (counts/min)	(% release)	Radioactivity (counts/min)	(% release)	Radioactivity (counts/min)	(% release)	Radioactivity (counts/min)	(% release)
Spleen	1	257	31·8	427	18·6	—	—	287	16·4
tissue	2	410	67·8	638	52·3	349	40·7	224	15·6
on grids	3	126	55·0	355	30·6	—	—	—	—
	4	204	35·8	103	15·5	—	—	200	10·0
	5	392	32·0	219	20·0	538	16·2	306	17·2
	6	158	22·0	182	10·8	—	—	383	9·3
	7	431	39·9	393	39·0	—	—	475	19·9
Spleen	8	184	14·3	273	4·4	—	—	137	12·5
cell	9	491	15·2	628	8·2	578	5·0	649	17·6
suspension	10	353	17·7	354	12·8	351	10·1	—	—

*Time of incubation 18 hr.

TABLE 5. Rat kidney cells incubated with lymphoid cells from rats, injected with different rat tissue extracts

Lymphoid cells from	Rats immunized with	Experiment 1				Experiment 2	
		3 hr incubation*		18 hr incubation		18 hr incubation	
		Radioactivity (counts/min)	(% release)	Radioactivity (counts/min)	(% release)	Radioactivity (counts/min)	(% release)
Blood	Kidney	—	—	89	34·5	232	22·4
	Liver	—	—	158	19·5	357	29·2
	Lung	—	—	—	—	326	16·4
	Adjuvant	—	—	196	7·3	262	9·1
Lymph nodes	Kidney	213	2·3	97	48·3	107	37·4
	Liver	315	0·2	160	25·5	125	42·4
	Lung	—	—	—	—	300	1·7
	Adjuvant	345	1·7	355	13·6	274	7·0
Spleen	Kidney	256	1·2	78	55·0	204	35·8
	Liver	366	0·5	126	55·0	126	34·1
	Lung	—	—	—	—	301	1·8
	Adjuvant	276	1·8	121	30·6	103	15·5
No lymphoid cells		—	—	—	—	200	10·0

*Complement added (10% guinea-pig serum).

injection. There was also a correlation between the intensity of the nephrosis and the outcome of the test. Thus, lymphoid cells from kidney immunized rats with moderate proteinuria (40–80 mg/day) never exerted any cytotoxic effects on kidney cells. The kidney cell cultures were 3–8 days old in most experiments. The 8-day-old cultures were as sensitive to the cytotoxic action of lymphocytes as the 3-day cultures were. All experiments with lymphoid cells presented in this article were performed under conditions which were optimal with respect to the variables just discussed.

Seven experiments were performed in which spleen tissue on metal grids was incubated with rat kidney cells for 18 hr (Table 4). As can be seen, in six out of seven experiments spleen cells from kidney sensitized rats gave rise to an isotope release which was 11·2–24·2% higher than that found in the controls with lymphoid cells from adjuvant immunized animals. The mean of the differences in Experiments 1–7 was 13·9 ± 2·8% (mean ± SEM). This was highly significant when tested with Student's *t*-test ($P<0{\cdot}005$). In Experiments 1–7 of Table 4, lymphoid cells from non-immunized rats were only used in two cases. In both, isotope release seemed to be slightly lower when normal spleen was used, and this was also seen in other experiments not presented here. However, so far the significance of this difference has not been investigated. On the other hand, addition of lymphoid cells, regardless of their origin, under these conditions led to a significant increase of cell damage seen in the absence of such cells (Table 4).

Table 4 also includes the results of three experiments in which suspensions of spleen cells were applied to the tissue cultures without grids. Lymphoid cells from nephrotic rats gave rise to an isotope release slightly higher than that caused by control lymphocytes. The significance of these results is doubtful.

In short-term experiments with rat kidney cells and lymphoid cells from nephrotic rats, cell damage was never seen. After incubation for 18 hr or more, lymphocytes from blood, lymph nodes or spleen of such animals were cytotoxic. In Experiment 1 of Table 5, lymphocytes from the same rat in each group were incubated with a kidney cell culture for 3 and 18 hr respectively. The release of isotope after 3 hr was insignificant in all cases. After 18 hr lymphoid cells from blood, lymph nodes and spleen of the kidney sensitized rats caused considerable cell damage.

Lymphoid cells from rats immunized with extracts of rat liver also damaged rat kidney cells. Two such experiments are seen in Table 5. Lymphocytes from lung sensitized rats (Table 4) had no, or only doubtful, effects. Lymphoid cells from spleen sensitized rats (not presented in this Table) were never cytotoxic when compared to the adjuvant sensitized controls.

As a further test of the specificity of the cytotoxic reaction lymphoid cells from nephrotic rats were incubated with target cells of different origin. Three typical experiments are shown in Table 6. Primary cultures of rat kidney and lung were susceptible to the cytotoxic action of these lymphoid cells. Cells from a suspension culture (established cell line) of rat kidney were also susceptible. On the other hand, established cell lines originating from parenchymal rat liver cells were resistent to the action of kidney sensitized rat lymphoid cells.

Experiments with Rat Sera

Primary rat kidney cultures on coverslips (4–8 days old) were labelled with ^{14}C-thymidine and incubated for 18 hr with 10% heat-inactivated rat serum. To some of the cultures 10%

TABLE 6. Lymphoid cells from rats incubated with different rat tissue cells*

Experiment No.	Target cells	Lymphoid cells	Rats immunized with	Radioactivity (counts/min)	Radioactivity (% release)
1	Rat kidney (primary)	Spleen	Kidney	158	22·0
		Spleen	Adjuvant	185	10·8
		—	—	554	9·3
	Rat lung (primary)	Spleen	Kidney	545	41·8
		Spleen	Adjuvant	1010	33·1
		—	—	3336	4·5
2	Rat kidney (cell strain)	Spleen	Kidney	453	18·1
		Spleen	Adjuvant	830	6·1
		Spleen	—	695	4·3
		—	—	933	5·7
3	Rat kidney (primary)	Lymph node	Kidney	145	47·3
		Lymph node	Adjuvant	98	26·8
		—	—	130	32·2
	Rat liver (cell strain)	Lymph node	Kidney	4150	41·6
		Lymph node	Adjuvant	3426	45·8
		—	—	3171	55·2

*Time of incubation 18 hr.

TABLE 7. Rat kidney cells incubated with sera from rats* immunized with rat kidney extracts or adjuvant

Sera from rats immunized with	Complement†	No. of sera	Radioactivity (% release) (Mean ± SD)
Rat kidney‡	—	11	6·7 ± 3·7
	+	5	7·9 ± 2·7
Adjuvant	—	9	6·3 ± 2·7
	+	3	7·5 ± 5·1

*Sera heat-inactivated for 30 min at 56°C. Concentration of serum 10%. Time of incubation 18 hr.

†10% guinea-pig serum.

‡Nephrotic rats.

complement (reconstituted freeze-dried guinea-pig serum, Sclavo, Siena, Italy), was added. The results are summarized in Table 7. Under these conditions no cytotoxic effects were observed with any of the twenty-eight sera tested. A few sera from nephrotic rats and from rats immunized with liver, lung or adjuvant only were also tested by incubation with kidney cells for 2 hr, in the presence of 10% complement. Cell damage was not observed. In order to search for the presence of antibodies in these sera, samples from eleven kidney immunized rats and from eleven control animals were tested for the presence of precipitating anti-rat kidney antibodies. With the Ouchterlony technique, four of the sera from the kidney immunized rats gave weak precipitates with lubrol or saline extracts of rat kidney. Sera from two animals, immunized with rat liver, sera from two lung sensitized rats and from seven adjuvant controls were negative. Aliquots of all sera were negative when tested with kidney antigen by the indirect haemagglutination technique. Rabbit anti-rat kidney sera, used as control, reacted strongly in both tests.

DISCUSSION

In experimental research concerned with cell-damaging immune reactions *in vitro*, isotopes have been exploited frequently by measuring the release of radioactivity from injured and labelled target cells. Most of the isotopes used for this purpose, such as ^{32}P-orthophosphate or ^{14}C-amino acids, are incorporated into the cytoplasm. Cells labelled in this way are subjected to a considerable spontaneous leakage of radioactivity (Perlmann & Broberger, 1963), which makes this type of labelling less appropriate for long-term experiments. In contrast ^{14}C-thymidine (or tritiated thymidine) is a marker of the stable DNA and the loss of isotope into the incubation medium is small. This method has therefore proved itself suitable for studies of lymphocyte induced cell damage *in vitro* (Vainio *et al.*, 1964; Holm, Perlmann & Werner, 1964). However, DNA is not released from damaged cells (Green *et al.*, 1959) unless the cells are treated with trypsin after contact with the injuring agents (Klein & Perlmann, 1963). This enzyme treatment affects the nuclei of damaged cells, while normal cells are left intact (Hirata, 1963). The mechanism of this action of trypsin is not known.

In this investigation the isotope released from the tissue culture cells, trypsinized after incubation with lymphoid cells, was used as a measure of cell damage. It was seen that this tended to give values which were slightly low, but it had the advantage of rapidity and simplicity. However, Klein & Perlmann (1963) found that the estimate of the number of dead cells in a suspension, when based on the determination of isotope release into Sup. II, correlates well with that obtained by supravital staining. This was also found in the present study (Table 2, and unpublished observations).

One would have anticipated finding more isotope in the medium, particularly when lymphoid cells were present, since autolytic changes, or enzymes released from the lymphocytes, could be expected to affect the nuclei of damaged cells in the same way as trypsin. Since the content of isotope in Sup. I was low, this was either not the case or isotope may have been re-utilized by other cells. It has been shown that human lymphocytes from peripheral blood take up isotope from dead ^{14}C-thymidine labelled cells, when synthesizing DNA. When no DNA was synthesized, re-utilization did not occur (unpublished observations). In the present experiments re-utilization of isotope would have tended to reduce

the sensitivity of the method. However, no re-utilization seemed to occur here since the addition of an excess of ^{12}C-thymidine in a few experiments at the beginning of incubation did not change the results (Holm & Perlmann, 1965).

Lymph node and spleen cells from rats with nephrosis damaged rat kidney cells in tissue culture, when incubated on metal grids according to Trowell (1959). In experiments with suspensions of spleen or lymph node cells, the cytotoxic effects were small or absent. In contrast, suspensions of blood lymphocytes were usually active. In general, rat lymphocytes have a short time of survival *in vitro* (Schrek, 1961). Under the experimental conditions used in this investigation, the survival rate of rat lymph node cells after 24 hr incubation was 30–40%. Incubation of tissue explants in close contact with the gas phase possibly facilitates the diffusion of oxygen into the cells, thus increasing their life span (Trowell, 1959). When using similar arrangements, Trowell (1959) was able to improve the survival of rat lymph node fragments considerably. This might explain the improved cytotoxic reactivity of rat lymphocytes, when incubated on grids. However, other unknown factors may be responsible for this.

Some evidence for the possible autoimmune character of the experimental nephrosis in rats is based on the findings of Heymann *et al.* (1959), who induced the disease in a small group of rats by immunization with autologous kidney extracts. It is also supported by the observations of Hess *et al.* (1962) and Heymann *et al.* (1962), who showed that experimental nephrosis could be transferred by injections of spleen cells from nephrotic rats to healthy recipient rats which had been made tolerant to the donor tissues. So far, however, reports concerning the induction of the disease in inbred rats have not been published. Our own attempts to induce nephrosis in highly inbred R-strain rats by long-term immunization with kidney extracts from syngeneic animals have failed so far (unpublished observations). Thus, the conditions for the development of experimental nephrosis have some resemblance to the findings, that autoimmune thyroiditis in rabbits (Weigle, 1965) and aspermatogenesis in guinea-pigs (Pokorná & Vojtíšková, 1964) can be induced by injections of chemically modified tissue specific antigens without adjuvant, but not with the native antigens. Moreover, autoantibodies were also produced after immunization with heterologous thyroglobulin (Weigle, 1965). In accordance with these observations the development of autoimmunity to kidney antigens in rats might be elicitated by immunization with kidney extracts of genetically foreign origin. However, the basis for the immunogenic differences between syngeneic and foreign kidney material is unknown.

Evidence has been brought forward for lymphoid cells from rats immunized with rat kidney causing injury of rat kidney cells. Such effects were also seen with cells from liver sensitized animals. This is not surprising in view of the fact that these organs share a larger number of antigens (Perlmann & Morgan, 1961). Sensitization with spleen, and to a certain extent also with lung, was ineffective. This underlines the organ specificity of the reaction. The specificity was tested further in the reverse experiments with various target cell types (Table 5). In some experiments primary cultures of rat lung cells as well as rat kidney cells were damaged. It is noteworthy that cells from the 5-month-old suspension culture of kidney cells were also damaged, in contrast to liver cells of similar origin, since organ specific antigens are usually lost relatively soon during cell culturing (Weiler, 1959; Brand & Syverton, 1962). More experiments are needed to establish this point.

An important initial step in the cytotoxic reactions of the present type is the aggregation of

lymphoid cells to the target cells. This has been observed in this, as well as in other, investigations (Rosenau & Moon, 1961; Koprowski & Fernandes, 1962). The reactions are strictly antigen specific (Rosenau & Moon, 1964) and can be blocked by pre-treatment of the target cells with specific antibodies (Möller, 1965b). Koprowski & Fernandes (1962) found that lymphocytes from adjuvant sensitized rats specifically aggregated to glial cells in monolayers, when pre-incubated with serum from rats with experimental allergic encephalitis. Similar experiments in the present system have so far been inconclusive (unpublished observations). Nevertheless, it is likely that antibodies or antibody-like substances on the lymphocytes from sensitized animals are mediating the necessary close contact between the cell types. How the ensuing cell damage is brought about remains to be established. It has recently been shown that aggregation of lymphocytes from normal animals to various target cells by means of phytohaemagglutinin may also give rise to target cell damage (Holm *et al.*, 1964; Bach & Hirschhorn, 1965; Möller, 1965a). This was only observed when the lymphoid cells were immunologically competent and were allogeneic or xenogeneic in regard to the target cells (Holm & Perlmann, 1965; Möller, 1965a). This suggests that the cytotoxic *in vitro* effects of lymphocytes are in some way connected to their ability to recognize 'foreign' histocompatibility antigens. Since lymphoid cells and kidney cells in the present investigation were also of allogeneic origin, the importance of histocompatibility antigens for the release of a target cell damaging response in the lymphocytes cannot yet be ruled out. Incubation of lymphoid cells from kidney sensitized animals with syngeneic kidney cells may throw light on this point.

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