Clin. exp. Immunol. (1970) **6,** 101–108.

SPECIFIC ANTISERA AGAINST RECIRCULATING AND NON-RECIRCULATING LYMPHOCYTES IN THE RAT

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(*Received* 28 *July* 1969)

SUMMARY

Heterologous anti-lymphocyte sera were prepared by injecting suspensions of recirculating or non-recirculating lymphocytes into rabbits. Recirculating lymphocytes were obtained from a thoracic duct fistula, and non-recirculating lymphocytes were obtained from the blood of rats in which thoracic duct lymph had been drained away for 3 days. The cytotoxic activity of the sera was assayed by measuring the isotope release from target cells labelled with ^{51}Cr. Antibodies specific for recirculating or non-recirculating lymphocytes could be demonstrated with the aid of cell adsorption techniques.

Cell-specific sera were used to estimate the proportion of recirculating and non-recirculating lymphocytes in lymphocyte suspensions obtained from thymus lymph nodes, blood and bone marrow. All thymocytes and most of the lymph node lymphocytes appeared to have antigenic properties in common with recirculating lymphocytes, whereas about 20% of the blood lymphocytes and most of the bone marrow lymphocytes belonged to the non-recirculating lymphocyte antigenic type.

INTRODUCTION

In the peripheral blood of rats there are at least two functionally different groups of lymphocytes, a major group which recirculates between blood and lymphoid tissue, and a minor group which does not recirculate (Iversen, 1969). Recirculating lymphocytes (RL) can be obtained from thoracic duct lymph and non-recirculating lymphocytes (NRL) from the blood of rats where prolonged lymph drainage has been carried out (Iversen, 1969).

The migration of RL through the endothelial cells of the lymph node venules appears to be a selective process (Marchesi & Gowans, 1964), which might depend on specific structures on the cell surface. It is not unlikely, therefore, that the two lymphocyte groups should also differ in their antigenic properties.

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In the present investigation antisera against RL and NRL were prepared in rabbits, which had been injected with either of these two cell types. After adsorption procedures the sera could be shown to contain specific antibodies against the two lymphocyte types. These specific sera were then used to estimate the proportion of RL and NRL in lymphocyte suspensions from thymus, lymph nodes, blood and bone marrow.

MATERIALS AND METHODS

Animals

Wistar rats weighing 200–300 g and albino rabbits weighing 2000–3000 g were used.

Anaesthetics

Either ether or intraperitoneally given pentobarbitone (Nembutal®, Abbott), 80 mg/kg body weight, was used during surgical procedures with rats.

Heparin

Heparin (A.L. Oslo, containing tri-cresol) was diluted to a final concentration of 10 i.u./ml.

EDTA-salt solution

This consisted of: 0·2 g disodium EDTA, 8·0 g NaCl, 0·2 g KCl, 1·15 g Na_2HPO_4, 0·2 g KH_2PO_4, 0·2 g glucose and distilled water to 1000 ml.

TBS (Tris-balanced salt solution)

Hanks's balanced salt solution devoid of bicarbonate: 9 volumes, and 0·2 M Tris–HCl buffer (pH 7·35): 1 volume.

Isopaque–Ficoll solution

A mixture, containing 10% Isopaque (Nyegaard & Co., Oslo) and 6·4% Ficoll (A.B. Pharmacia, Uppsala) in water was used.

Radioactive material

$Na_2{}^{51}CrO_4$, specific activity 50–150 mCi/mg Cr (Institutt for Atomenergi, Kjeller, Norway).

Cell counts

Cell counts were carried out with an electronic particle counter (Celloscope 101, A.B. Ljungberg, Stockholm, Sweden) using a Cetrimide diluting solution (Kvarstein, 1967).

Differential counts

These were made on smears stained with May–Grünwald–Giemsa.

Cell suspensions

Cell suspensions of RL and NRL were prepared as previously described (Iversen, 1969).

RL was obtained from thoracic duct lymph and NRL from the blood of rats where lymph drainage had been carried out for 3 days.

Pure lymphocyte suspensions were prepared by a gradient layer centrifugation (Bøyum, 1968). Blood suspensions were carefully layered on top of the Isopaque–Ficoll solution in centrifuge tubes. Centrifugation for 40 min at 400 ***g*** caused sedimentation of granulocytes and erythrocytes, whilst the mononuclear cells remained at the fluid interface. Cell suspensions obtained from this interphase contain 90–95% lymphocytes contaminated by granulocytes and thrombocytes.

Granulocyte suspensions used for the adsorption procedure were prepared in the following way. The sediment from the gradient layer centrifugation was resuspended in EDTA-salt solution to which was added one-tenth the volume of a 0·1% methylcellulose solution (Metocel 25 CP Fluca AG, Switzerland). This caused aggregation and sedimentation of the major part of the erythrocytes. Hyposmotic haemolysis, induced by treating the supernatant cells with 0·25% NaCl for 1 min, removed most of the remaining erythrocytes. A granulocyte suspension contaminated by 10–20% mononuclear cells was obtained in this way.

Preparation of antisera

Portions of RL and NRL suspensions, which contained 5×10^6 to 5×10^7 cells, were washed twice with Hanks's solution, and each of the portions was then given as weekly intravenous injections to one rabbit. Seven days after the fifth injection the rabbits were bled and sera prepared. Both types of sera were inactivated by heating to 56°C for 30 min, and they were then adsorbed with erythrocytes until all haemagglutinating activity had been removed.

Cytotoxic assay

Cytotoxic activity of the sera against various leucocytes was assayed by measuring the isotope release from target cells labelled with ^{51}Cr. The method was adopted from Sanderson (1964). Fresh guinea-pig serum was used as complement. The cells were labelled by incubating 100 μCi $Na_2{}^{51}CrO_4$ and 10^6–10^7 cells suspended in 0·5 ml TBS for 30 min at 37°C. The cells were washed three times in TBS, resuspended and diluted in TBS to a cell concentration of 5×10^5/ml. Serial doubling dilutions of antisera with TBS were then made. One hundred microlitres of diluted serum, 50 μl of cell suspension and 50 μl of guinea-pig serum were incubated for 30 min at 37°C. The cytolytic process was terminated by adding 1·5 ml TBS at 4°C and the cells spun down by centrifugation at 4°C for 10 min at 1000 ***g***. The supernatant was removed and the radioactivity of the cell button was measured in a Packard Well-Type Scintillation Counter.

Control mixtures without antiserum and without guinea-pig serum but adjusted to the standard volume with TBS were always included. The highest dilution of the antiserum which gave more than 50% cell lysis was recorded as the cytotoxic titre. The use of such an end point for the titration will theoretically give the most sensitive assay (von Krogh, 1916), and an additional advantage is the negligible influence on this titre by irrelevant cells contaminating the target cell suspension. An estimate of the amount of isotope released from the cells at 100% lysis was required to calculate the values corresponding to 50% lysis. Non-immune lysis, effected by freezing and thawing of the cells, was found unsuitable for this purpose, because the isotope release by this procedure varied considerably and would also affect non-target cells. Therefore, the plateau level of isotope release caused by high concentrations of an effective antiserum was assumed to reflect 100% lysis of the target cells.

As a rule this value of isotope release was as high or even higher than that obtained by non-immune lysis. The titration curves of two different antisera are shown in Fig. 1; it is apparent from the figure how the 100% lysis value is estimated.

The guinea-pig serum had no cytolytic activity against leucocytes, but caused haemolysis of contaminating erythrocytes when these were present. This did not ordinarily interfere with the reproducibility of the cytotoxic assay.

The influence on the assay of thrombocytes present in lymphocyte suspensions obtained from blood was also evaluated. The use of lymphocyte suspensions prepared from defibrinated blood, where most of the thrombocytes had been removed, gave the same cytotoxic titre as did lymphocyte suspensions prepared from heparinized blood.

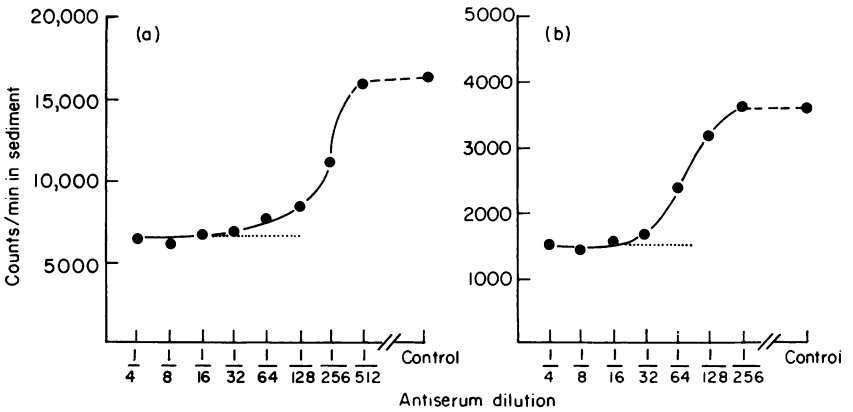

FIG. 1. Titration curves of the two antisera used to estimate the proportion of lymphocytes of RL-type and NRL-type in different tissues. Counts/min in sediment is the radioactivity in cell sediment after incubation of ^{51}Cr-labelled target cells with diluted antiserum and guinea-pig serum. The plateau level caused by high concentrations of antiserum was assumed to reflect 100% lysis of target cells (dotted line). (a) Anti-RL-serum adsorbed with erythrocytes and granulocytes titrated against RL. (b) Anti-NRL-serum adsorbed with erythrocytes and RL titrated against NRL.

Adsorption procedures

To remove unspecific antibodies the antisera were adsorbed with a certain amount of cells of other types than the actual target cells. The cells used were washed in TBS and then incubated with undiluted antisera for 1 hr at 20°C, whereafter they were removed from the sera by centrifugation.

RESULTS

The cytotoxic activity of anti-recirculating lymphocyte serum (a-RL-serum) which had been adsorbed with erythrocytes, was tested against different leucocyte suspensions (Table 1). In addition to a high activity against RL, lower activities against NRL and granulocytes were recorded. The latter activities were completely removed by adsorption with granulocytes, so that a serum specific against RL was obtained. The latter activity was completely removed by adsorption with RL.

The cytotoxic activity of an erythrocyte-adsorbed anti-non-recirculating lymphocyte

serum (a-NRL-serum) is shown in Table 2. The high activity recorded against RL was completely removed by adsorption with these cells. After this procedure the activity against NRL and granulocytes was considerably reduced but not eliminated. By further adsorption with either NRL or granulocytes a slight but significant activity against each of these two

TABLE 1. Cytotoxic activity against rat leucocytes of anti-RL serum adsorbed with erythrocytes, given as reciprocal titres

Additional adsorption	Target cells		
	RL	NRL	Gran
None	1024	64	32
Adsorbed with 3×10^7 gran/ml	512	< 2	< 2
Adsorbed with 3×10^7 gran/ml + 1×10^9 RL/ml	< 2	< 2	< 2

The cytotoxic assay was based on isotope liberation from ^{51}Cr-labelled target cells. The highest dilution of the antiserum which gave more than 50% cell lysis was recorded as cytotoxic titre.

RL, Recirculating lymphocytes; NRL, non-recirculating lymphocytes; gran, granulocytes.

cell groups could be demonstrated. The cytotoxic activity of this antiserum against NRL could be completely removed by adsorption with these cells.

The cell-specific sera obtained by these adsorption procedures were used to estimate the proportion of RL and NRL in lymphocyte-rich cell suspensions obtained from several

TABLE 2. Cytotoxic activity against rat leucocytes of anti-NRL serum adsorbed with erythrocytes, given as reciprocal titres

Additional adsorption	Target cells		
	RL	NRL	Gran
None	1024	256	128
Adsorbed with 3×10^8 RL/ml	< 2	32	32
Adsorbed with 3×10^8 RL/ml + 7×10^7 gran/ml	< 2	16	< 2
Adsorbed with 3×10^8 RL/ml + 1×10^7 NRL/ml	< 2	< 2	32

The cytotoxic assay was based on isotope liberation from ^{51}Cr-labelled target cells. The highest dilution of the antiserum which gave more than 50% lysis was recorded as the cytotoxic titre. Symbols as Table 1.

sources, namely thymus, lymph nodes, blood and bone marrow. Lymphocyte suspensions from the blood of an animal where no lymph drainage had been carried out was obtained as described in 'Materials and methods'. Thymus, samples of mesenteric lymph nodes, and of bone marrow, were dissected out and minced in EDTA-salt solution and then passed through a fine-meshed brass filter. The cells were then separated by the gradient layer

centrifugation method described in 'Materials and methods'. Lymphocyte-rich cell suspensions obtained from the gradient interface were labelled with ^{51}Cr and incubated with either one or both of the two antisera, and the same procedure was followed as when recording the cytotoxic titre. The antiserum dilutions now used were sixteen times more concentrated than that required for 50% lysis. These sera were assumed to give near 100% lysis of their specific target cells, and since they had been adsorbed, the sera were presumed not to affect the other lymphocyte type to a significant degree. The ratio between the isotope amount liberated by the most effective antiserum and that liberated by the two antisera together, was assumed to reflect how big a fraction of all the lysed cells the predominating lymphocyte type amounted to. The results given in Table 3 indicate that all thymocytes have antigenic

TABLE 3. Amounts of recirculating lymphocyte type calculated as percent of all lymphocytes present in lymphocyte-rich suspensions obtained from different tissues

Cell source	Thymus	Lymph nodes	Blood	Bone marrow
No. of animals	4	3	4	4
Mean percentage of RL type	100	92	84	8
Range (%)	100–100	76–100	62–100	0–18
Mean percentage of lymphocytes in cell suspension	100	95	95	45

Cell suspensions prepared and cells labelled with $Na^{51}{}_2CrO_4$ as described in text. Evaluation of relative RL and NRL amounts based on isotope liberation from the various cell suspensions when exposed to anti-RL and/or anti-NRL sera. For details see text.

properties in common with RL. Most of the lymph node lymphocytes belong to the same antigenic type. As expected from lymph drainage studies (Iversen, 1969), the proportion between RL and NRL in blood seemed to be about 4:1. Most of the bone marrow lymphocytes appeared to belong to the NRL type.

DISCUSSION

The effects of the present rabbit anti-rat leucocyte sera have demonstrated that there are specific antigenic structures on RL and NRL. The antiserum against RL had a cytotoxic titre comparable to that found with the similarly prepared anti-lymphocyte sera of other workers (James & Anderson, 1967). The cytotoxic activity of the present serum against NRL and granulocytes might be due to contamination with these cell types of the cell suspension used for immunization. This possibility is, however, not very likely, since thoracic duct lymph is supposed to contain almost exclusively recirculating lymphocytes. A cross-reaction due to common antigens on the three leucocyte types seems a more probable explanation. This is in accordance with the observation of Thorsby (1967) that human lymphocytes and granulocytes possess both common and cell specific antigens.

The fact that pure suspensions of neither NRL nor granulocytes were available makes the

interpretation of the a-NRL serum titres difficult. The NRL-cell suspensions used for immunization were contaminated with granulocytes and probably also with RL. The high cytotoxic titre of the a-NRL serum against RL can thus be explained on the basis of such a contamination combined with a stronger antigenicity of RL than of the other leucocytes. The cell suspensions used for adsorption were also contaminated with small amounts of the other cell types. It is thus not possible on the basis of the present investigation to decide whether the multipotency of the a-NRL serum is due to impure stimulation or to the existence of antigens common for the various leucocyte types dealt with. On the other hand, during titre evaluation impurities of the target cell suspensions will be of a minor importance. If the proportion of contaminating cells are kept below 30–35%, these impurities will not interfere seriously with the cytotoxic assay, since this is based on 50% lysis of all antiserum susceptible cells present. Small amounts of antibodies are thus not recorded by the titration. In this respect the adsorbed sera have, therefore, been made specific against each of the two lymphocyte groups, even if they still contain small amounts of antibodies against the other lymphocyte type. The estimate of the NRL–RL ratios in different tissues (Table 3) will probably not be very precise though. These estimates also depend on the questionable assumption that all cell types tolerate the preparative procedure equally well, and that the isotope release on cell lysis is equally marked for all cell types. Nevertheless, the estimates indicate which one of the two lymphocyte types is the predominating one in the tissues investigated.

On the basis of observations on neonatally thymectomized animals (Miller & Osoba, 1967) and studies of the migration of labelled thymocytes (Weissman, 1967), a thymic origin of the RL has been proposed. The present finding of common antigens on thymocytes and RL is in accordance with this hypothesis. The distribution pattern of the NRL-antigenic lymphocyte type, on the other hand, points to a bone marrow origin of these cells.

What then is the physiological significance of specific antigenic structures on surface membranes of blood cells? The present investigation indicates that they might be important for the route of circulation and the homing of different cell groups.

ACKNOWLEDGMENTS

Financial supports from The Nansen Foundation, The Norwegian Council on Cardiovascular Diseases, Anders Jahres Fund for the promotion of Science and The Norwegian Research Council for Science and the Humanities are gratefully acknowledged.

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