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The Distribution of Rabbit Anti-Mouse Thymus Globulin After Injection into Mice* (34025)

CARLOS NAVA, H. O. O'KANE, AND R. G. SHORTER

Section of Tissue & Organ Transplantation, Mayo Clinic & Foundation, Rochester, Minnesota

The immunosuppressive action of heterologous antilymphoid sera (ALS) has been well established in the laboratory in various species of animal, and prolongation of survival of allografts of skin (1), kidney (2), and liver (3) and xenografts of tumor (4) has been achieved with their use. Such observations have led to the clinical trial of ALS as an adjunct to more conventional immunosuppressive therapy in the grafting of organs in man (5, 6). Although theories have been formulated as to the mechanism of the immunosuppressive action (7), no definitive explanation has been found. We considered that a study of the distribution of heterologous antilymphoid globulin *in vivo* after intravenous injection might be contributory in this regard and the distributions of radio labeled gamma globulin and IgG prepared from heterologous antithymus serum were investigated after their parenteral injection. Presented here is a report of the results of these experiments.

Materials and Methods. Rabbit anti-mouse thymus sera (RAMTS) were prepared by injecting intravenously each of three New Zealand rabbits (average weight 2 kg) with suspensions of 1×10^9 viable thymus cells obtained from neonatal mice. Each rabbit was injected twice at a 2-weekly interval and bled 1 week after the second injection. The sera were pooled and heated at 56° for 30 min. The initial titer of lymphocytotoxicity *in vitro* for murine lymphocytes was 1:4096 when tested by the method described by Terasaki and associates (8) and titrated to less than 10% kill. After repeated absorptions with murine red cells (1 vol erythrocytes: 4 vol serum) to remove *in vitro* hemagglutinating and hemolyzing activity, and a single absorption with a homogenate of murine kidney (1 vol kidney homogenate: 4 vol serum), the gamma globulin (ATG) was precipitated with 40% ammonium sulfate.

In preparation for labeling with radioactive iodine, one portion of the precipitate was reconstituted in sodium phosphate buffer, 0.05 *M*, pH 7.0, to the original volume of the serum and then dialyzed against the same buffer. At this stage the lymphocytotoxic titer of the globulin (ATG) was 1:4096 when tested *in vitro* against murine lymphocytes from the peripheral blood and titrated to less than 10% kill. After reconstitution in 0.1 *M* potassium phosphate buffer (pH 8.0) another portion of the precipitate was dialyzed against this same buffer prior to the separation of the IgG (ATIgG) by column chromatography using diethylaminoethanol (DEAE) cellulose (9). The immunoelectrophoretic pattern of this fraction showed the presence

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of a single band of precipitation located in the position normally ascribed to IgG.

For control purposes, sera were obtained from nonimmunized New Zealand rabbits, pooled, and processed in a similar fashion to yield gamma globulin (NRG) and IgG, (NIgG). No lymphocytotoxic activity was detected in these control materials.

Each fraction from the RAMTS was injected by the intravenous route simultaneously with the administration of the corresponding fraction from normal serum and the study of the distribution *in vivo* of these different fractions involved the use of a double radio labeling technique. Accordingly, the gamma globulin and the IgG from sera of rabbits immunized with murine thymocytes were labeled with ^{125}I[1] and the gamma globulin and the IgG from sera of nonimmunized rabbits were labeled with ^{131}I[1] by the method of McConahey and Dixon (10).

In an attempt to isolate antilymphoid antibodies with a high localization specificity for lymphoid tissues, eluates from the RAMTS and the normal serum were prepared by a method described by Korngold and Pressman (11). Aliquots of rabbit anti-mouse thymic globulin labeled with ^{125}I (RAMTG-^{125}I), or normal rabbit globulin labeled with ^{131}I (NRG-^{131}I), containing equal amounts of protein, were absorbed to equal weights of lyophilized murine thymic tissue and then eluted by heat at 60° for 15 min. Immunoelectrophoresis of the eluates from RAMTG or NRG showed the presence of a single precipitation band located in the IgG position. The lymphocytotoxic titer of IgG prepared from the RAMTG by this method was 1:128 while there was no cytotoxic activity in the supernatant fraction remaining after the RAMTG-^{125}I had been absorbed with the murine thymic tissue. No lymphocytotoxic activity was demonstrable in the IgG or in the supernatant fraction obtained after the absorption of normal rabbit serum with murine thymic tissue. On analytical ultracentrifugation in a Beckman model E analytical ultracentrifuge, the IgG prepared by chromatography from either serum was homogenous with a sedimentation velocity of 7.0S and the IgG absorbed and eluted from RAMTG or NRG also showed a sedimentation coefficient of 7.0S.

The immunosuppressive properties of gamma globulin, IgG obtained from the column and IgG obtained by absorption and elution from the RAMTS were confirmed individually by their ability to produce significant prolongation of skin allografts from CBA mice when administered subcutaneously daily to groups of six C57BL/6 mice. No immunosuppressive effects were shown by the preparations from normal rabbit serum when tested in the same allograft system.

After their thyroid glands had been blocked with inorganic iodide each of six C57BL/6 mice (13–20 g body weight) was given a single intravenous injection of a mixture containing equal weights (0.104 mg) of RAMTG-^{125}I and NRG-^{131}I. Twenty-four hours later each animal was injected intravenously with human albumin tagged with ^{99m}TC (12) 0.2/μCi activity to enable a correction subsequently to be made for the amount of blood contained in each tissue sample.

After allowing a 4-min period for equilibration, the mice were killed instantaneously and samples of blood, thymus, lymph node, spleen, small intestine (Peyer's patches), liver, kidney, adrenal, and lung were taken and counted immediately for radioactivity of ^{99m}TC and ^{131}I using a two-channel scintillation counter (Autowell II).[2] After 3 days, to permit the decay of ^{99m}TC, all the samples were recounted for radioactivity of ^{125}I and ^{131}I.

The weight of blood in any particular tissue sample was obtained by dividing the counts per minute of ^{99m}TC from the sample by the counts per minute of ^{99m}TC from a gram of blood. The weight of blood then was subtracted from the total weight of the tissue sample to give a net tissue weight. When the weight of blood present in each tissue had been estimated, and knowing the values for the radioactivity of ^{131}I and ^{125}I per gram in

[1] Carrier-free ^{125}I and ^{131}I (Iso/Serve, Cambridge, Mass.)

[2] Picker Nuclear, White Plains, N. Y.

blood, a correction then was made for the radioactivity of ^{125}I and ^{131}I resulting from their presence in the blood in each organ sample. When this correction was applied to the counts from each tissue sample, the activity of ^{125}I and ^{131}I in the tissue substance alone could be obtained.

The radioactivity in ^{125}I and ^{131}I per gram in each of the tissues was expressed as a percentage of the total dose of each isotope injected and the net localization of ATG^{125}I was obtained by subtracting the corrected value of the counts of NRG^{131}I from those of RAMTG-^{125}I.

A similar method was used to study the distribution of 0.055 mg of RAMTIgG^{125}I ATIgG and NIgG-^{131}I after injection into a group of six C57BL/6 mice (12–19 g body weight).

In the studies of the distribution of IgG obtained from the RAMTS or normal serum by absorption with thymic tissue, the method employed was the same but the injected amounts of the IgG from the RAMTS and the normal rabbit serum differed since although equal amounts of globulin from each serum were absorbed with equal weights of thymic tissue the resulting amounts of IgG obtained after elution were not equal. As a result, 60 μg of RAMTS IgG (eluted) labeled with ^{125}I, and 14 μg of IgG (eluted) from the normal serum, labeled with ^{131}I, were injected simultaneously into each of six C57BL/6 mice (11–21 g). For each experiment in which eluted IgG was used the results also were expressed as a ratio of the net localization of the RAMTIgG (eluted) ^{125}I to the NRIgG (eluted) ^{131}I and this was termed the uptake ratio.

In each group of experiments the results were normalized to 20-g mice.

Results. Twenty-four hours after their injection into normal mice, the distributions of RAMTG-^{125}I and RAMTIgG-^{125}I did not differ significantly (p 0.1) from those of NRG-^{131}I and NRIgG-^{131}I in any of the organs examined. In contrast, 24 hr after the injection of IgG prepared by absorption with thymic tissue followed by elution, there was a significant difference ($p<0.05$) between the distribution of that obtained from the antithymus serum and that of the IgG obtained from normal serum since a greater degree of localization of the IgG from the RAMTS was demonstrable in the thymus, lymph node, spleen, and lung. If the results of the localization in the different sites were expressed as the ratio of net localization of the IgG absorbed from the RAMTG to that obtained from NRG, then the thymus showed the highest uptake ratio. The results are summarized in Table I.

Discussion. Denman and Frenkel (13) reported that injected heterologous antilymphocyte globulin reached the lymph nodes and spleen in limited amounts and that its access to the thymus was even more limited. From their observations these workers concluded that the uptake in lymphoid tissues was no higher than in other tissues. However, the correction used for the radioactivity arising from activity in the blood did not take into account the great natural variation of blood content from tissue to tissue and radiolabeled normal rabbit IgG was not included as a control so these differences in technique make it difficult directly to compare their results with ours.

In contrast to the findings of Denman and Frenkel, Hintz and Weber (14), reported that the greatest degree of localization of injected radiolabeled heterologous antithymic globulin was in the thymus gland. These workers applied a more precise correction for the activity resulting from the presence of blood in each tissue but did not compare the distribution of the antithymic globulin with that of globulin from normal rabbit serum.

Using a paired-label technique, Guttman and co-workers (15) compared the distribution *in vivo* of ^{125}I-labeled IgG from normal rabbit serum with that of ^{131}I-labeled IgG from rabbit anti-rat thymus serum after injection into inbred rats which had received renal allografts. They found selective localization of the anti-rat thymus IgG in the grafted kidney and in the spleen but none in the thymus or other lymphoid tissues. These authors did not mention that any correction was used for the radioactivity originating

TABLE I. Net Localization and Uptake Quotients in Mouse Tissues 24 Hours after Intravenous Injection of Labeled IgG Absorbed by Thymic Tissue from Rabbit Anti-Mouse Thymus Sera and Normal Rabbit Sera.

Tissue sample	ATIgG (eluted)[a] ^{125}I (% injected dose/g)	NRIgG (eluted)[a] ^{131}I (% injected dose/g)	Net % of injected dose/g[b]	Uptake ratio[c]
Thymus	0.4157[d]	0.1238[d]	0.2919	2.3
Lymph node	0.5329[d]	0.2511[d]	0.2818	1.1
Spleen	0.4987[d]	0.2437[d]	0.2550	1.0
Lung	0.4037[d]	0.1987[d]	0.2050	1.0
Liver	0.3097	0.1548	0.1549	1.0
Skin	0.4194	0.2435	0.1759	0.72
Adrenal	0.4132	0.2554	0.1578	0.61
Small intestine	0.2371	0.1183	0.1188	1.0
Kidney	0.3984	0.2897	0.1087	0.37
Brain	0.0141	0.0116	0.0025	0.21

[a] Mean value for six mice.

[b] Net values obtained by subtracting value obtained from NRIgG (eluted) ^{131}I from the value obtained from ATIgG (eluted) ^{125}I.

[c] Uptake ratio

$$= \frac{\text{ATIgG (eluted) } ^{125}I \text{ \% injected dose/g} - \text{NRIgG (eluted) } ^{131}I \text{ \% injected dose/g}}{\text{NRIgG (eluted) } ^{131}I \text{ \% injected dose/g}}.$$

[d] Significant difference (p 0.05).

from the blood in the various organ samples, an omission which makes it difficult critically to evaluate their findings. However, from their observations, these authors speculated on the possible local action of LAS at the graft site as a factor in achieving its immunosuppressive effects.

In the present study no significant differences were found between the distributions *in vivo* of gamma globulin, or IgG prepared by column chromatography, from heterologous antilymphoid serum and those of similar fractions derived from heterologous normal serum 24 hr after intravenous injection into mice. It was considered that this inabilitity to demonstrate any specific tissue-localizing properties of these materials could have been due simply to the very small amounts of specific tissue-localizing antibodies present in the injected materials and the insensitivity of the method to detect such small quantities. This conclusion was supported by the findings of Woodruff and co-workers (16) which indicated that 95% of radiolabeled IgG prepared by chromatography from a horse anti-human lymphoid serum failed to combine with human lymphocytes *in vitro*, suggesting that only a very small fraction of the anti-lymphoid serum was capable of specific localization in lymphoid tissues. This train of thought led us to use IgG prepared by absorption of anti-lymphoid globulin with thymic tissue since this was considered to be more likely to demonstrate selective localization in lymphoid tissues and was shown to be immunosuppressive in the skin allograft system in mice.

Our results are in agreement with those of Hintz and Weber in that the greatest degree of localization of heterologous IgG occurred in the thymus. This finding might support the concept that at least part of the action of ALS is mediated through the thymus, resulting in a slowing down of the recruitment of immunocompetent cells into the circulatory lymphoid pool (17).

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Myocardial Metabolism IV. Metabolism of Free and Esterified Cholesterol by the Perfused Rat Heart and Homogenates* (34026)

S. L. Gartner and George V. Vahouny

Department of Biochemistry, School of Medicine, The George Washington University, Washington, D. C. 20005

It is well documented (1) that circulating fatty acids provide an important energy source to the myocardium, and several studies have been directed to the importance of albumin-bound free fatty acids in this respect (2–5). More recent investigations have extended the early observation of Bing *et al* (6) which indicated that a more important source of fatty acids was derived from circulating esterified lipids which comprise over 90% of the total circulating lipid. These studies (7–12) have revealed that rabbit and rat heart can extract and oxidize triglyceride fatty acid from density < 1.006 lipoproteins; this was also the case for triglyceride of the density 1.006–1.070 lipoproteins, but to a lesser extent (9). Phospholipids from all lipoprotein fractions were not extracted by isolated perfused rat heart and were, therefore, not an energy source to this tissue (9).

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Miller *et al.* (13) also reported that during perfusion of rabbit hearts with hypercholesteremic plasma, there was an increase in circulating free cholesterol with a proportional loss in esterified cholesterol. These studies were carried out under hypoxic conditions and, when oxgenated red blood cells were added to the perfusing diluted serum, less dramatic results were obtained. Also, Delcher *et al.* (9) were not able to ascertain whether the perfused rat heart could extract or utilize circulating free and esterified cholesterol.

It was the purpose of the present study to determine whether (1) the isolated perfused rat heart is able to extract labeled cholesterol and cholesterol palmitate; (2) cholesterol ester fatty acid is oxidized; (3) heart contains cholesterol ester hydrolase and/or low- or high-energy cholesterol-esterifying system; and (4) the extent of cholesterol biosynthesis from labeled mevalonate.

Materials and Methods. Cholesterol-4-^{14}C,