

# Effect of ribonucleic acid perfusion on canine kidney and liver homograft survival

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**M**any of the problems of organ transplantation could be minimized if it were possible to mitigate graft rejection by modifying the transplanted tissue rather than the host immunologic response. Efforts to achieve this objective have been unsuccessful, with occasional possible exceptions,<sup>4, 9, 11</sup> of which the most intriguing was described by Jolley, Hinshaw, and Peterson.<sup>11</sup> They reported that rabbit skin grafts which were first immersed in homologous ribonucleic acid (RNA) and then transplanted to recipient animals which were given intravenous RNA had a survival 4 times longer than controls. The role of the preliminary soaking was not analyzable in these experiments, but the authors also reported that human skin homografts subjected only to RNA soaking had unusually protracted viability when placed upon patients with burns.<sup>10</sup> Similar attempts to "pretreat" whole organ homografts have so far been unsuccessful.<sup>21</sup>

In our experiments, this concept has been

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examined in dogs by perfusing kidney and liver homografts with RNA prepared from the spleens of the prospective recipients (autologous RNA) or other dogs (homologous RNA). After transplantation to unmodified recipients, about one fourth of these life-sustaining organs had a definitely prolonged functional survival.

## METHODS

Mongrel dogs weighing 10.5 to 25.9 kilograms were used. Anesthesia was with sodium pentobarbital supplemented with the tranquilizer phencyclidine hydrochloride (Sernylan). Litter mates were never used, and donor-recipient combinations were selected on the basis of dissimilar appearance of the animals. Renal homotransplantation was to the standard pelvic site and the animals' own kidneys were removed at the same operation. Orthotopic liver transplantation was performed after total recipient hepatectomy. After both kinds of transplantation, antibiotics were given. No immunosuppressive agents were used. Recipients which died in less than 5 days because of homograft vascular thrombosis, intussusception, or pneumonitis were excluded; further mention will be made only of the retained experiments.

This did not bias the results, since the loss rate was 10 of 40 (25 percent) in the combined control groups as compared to 16 of 56 (28 percent) in the test series.

The criteria used to evaluate the results included survival times, the quality of organ function just prior to death, and histologic studies of the retrieved homografts. For the last purpose, all tissues were fixed at the time of autopsy in 10 percent formalin. For survival computation, maximum credit for individual dogs was limited to 70 days.

RNA was prepared from nonwashed, quick-frozen dog spleens by phenol extraction. The method of Scherrer and Darnell<sup>22</sup> was used, except that bentonite added to a final concentration of 0.04 percent was used instead of polyvinyl sulfate as a ribonuclease inhibitor and re-extraction was done only once. The final precipitate had the ultraviolet absorption spectrum characteristic for RNA with a maximum at 260 m $\mu$  and a 280:260 m $\mu$  ratio of 1:1.72 to 1:2.34 (mean, 1.98). The DNA content was less than 2.0 percent measured with the diphenylamine method and the protein content was lower than 0.1 percent as determined by the phenol biuret reaction. Before use, the frozen RNA precipitate was dissolved to a concentration of 0.5 to 1.6 mg. per milliliter in cold lactated Ringer's solution (5° C.) which contained 5 mg. per 100 ml. procaine and enough phosphate buffer to adjust the pH to 7.45 to 7.55. In various test groups other constituents were added as outlined below.

The general technique of perfusion was the same in all the test and control groups. The excised organs were first cleared of blood by intravascular infusion of cold (5° C.) lactated Ringer's solution. This was followed by 50 and 100 ml. of the chilled special solutions for the kidneys and livers, respectively. At the completion of perfusion, the kidneys were placed in a refrigerator at 5° C. for 15 minutes and then transplanted to their new host. Transplantation of the livers was started immediately. Thus, exposure to the portion of the test solutions which remained in the organs was variable, ranging from 35 to 65 minutes. Ischemia times for the kidneys

ranged from 43 to 75 minutes and for the livers from 51 to 64 minutes.

## MATERIAL

The experimental and control series all involved renal transplantation except for Group 2B. They were divided as follows:

**Group 1. Autologous RNA (10 dogs).** The spleens of the eventual recipients were removed 2 to 5 days before transplantation and used as the RNA source (Fig. 1) for renal homograft perfusion.

**Group 2. Autologous RNA plus DEAE-dextran (17 dogs).** The perfusate was the same as in Group 1 except that high molecular weight DEAE-dextran\* was added. This change was made because of the reports that DEAE-dextran enhances the infectivity of viral RNA.<sup>20</sup> (A) Renal homografts (10 dogs), 1.0 mg. per milliliter DEAE-dextran was added; and (B) liver homografts (7 dogs), 0.3 mg. per milliliter DEAE-dextran was added.

**Group 3. Homologous RNA (10 dogs).** The RNA was prepared from the spleen of a different dog than the homograft recipient or donor (Fig. 1). Otherwise the renal homograft perfusion was as in Group 1. Recipient splenectomy was performed at the time of transplantation.

**Group 4. Homologous RNA plus DEAE-dextran (10 dogs).** The experiments were as in Group 3 except that 1.0 mg. per milliliter DEAE-dextran was added to the perfusate.

**Group 5. Control. Homologous RNA plus ribonuclease (10 dogs).** One hour before the experiment, 5  $\mu$ g commercial ribonuclease† (RNase) per 1.0 mg. RNA was added to the perfusate at room temperature. Otherwise, the procedure was as in Group 3.

**Group 6. Control. DEAE-dextran (10 dogs).** The same dextran-containing perfusate was used as in Groups 2 and 4, except that RNA was omitted.

**Group 7. Control. No additive (10 dogs).**

\*Diethylaminoethyl-dextran prepared from dextran, Mw =  $2 \times 10^6$ , Pharmacia, Piscataway, N. J.

†Beef pancreas ribonuclease, Worthington Biochemical Corporation, Freehold, N. J.

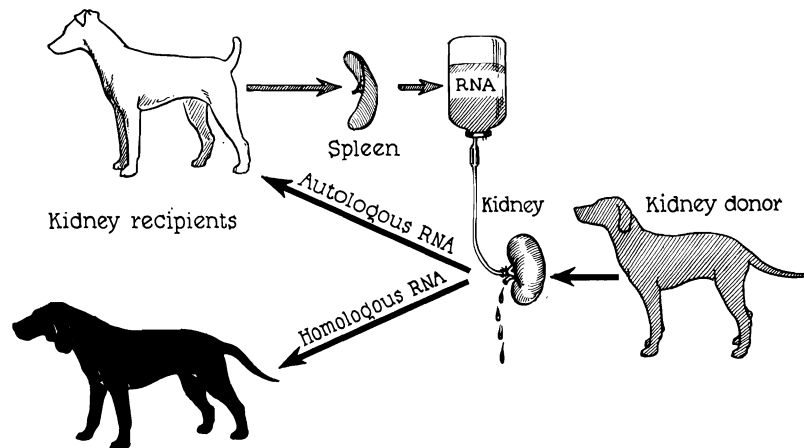


Fig. 1. Treatment of renal homografts with RNA.

Homograft perfusion was with unmodified lactated Ringer's solution; ischemia times and other procedural details were as in Groups 1 to 6.

**Group 8. Homologous RNA and renal autotransplantation (12 dogs).** The kidneys were removed, perfused with homologous RNA solution as in Group 3, and transplanted to the standard pelvic site. Splenectomy was performed at the same operation.

## RESULTS

**Survival and function.** The results with renal homotransplantation are summarized in Table I. When the homografts were perfused with the 3 control solutions (Groups 5 to 7), one recipient animal in each series of 10 lived for more than 20 days, the maximum being 44 days. Mean survival in the 3 groups ranged from 12.6 to 13.4 days. Uremia developed in every instance.

In contrast, there were several examples of prolonged survival in each series in which the homografts were treated with RNA (Groups 1 to 4), and the mean survival was increased to 17.6 to 24.2 days. The results were comparable with autologous and homologous RNA and were not significantly influenced by the addition of DEAE-dextran to the perfusate. Of the 40 recipients in the combined test groups, 17, 11, 8, and 5 lived for more than 15, 20, 30, and 40 days, re-

spectively. The longest survival was 123 days; in this animal the BUN was less than 35 mg. percent during the first 90 postoperative days (Fig. 2). In 5 other animals which died of pneumonitis and wasting after 11, 16, 21, 33, and 55 days, BUN's determined from 1 to 3 days before death were normal. The remaining 35 animals developed uremia. When compared to all the control series, the prolongation in survival (Table I) in the combined test groups was statistically significant ( $p < 0.025$ ).

Nine of the 12 dogs in which renal autografts were first perfused with homologous RNA (Group 8) lived until being killed 8 to 79 days postoperatively. At the time of killing all 9 animals had a normal BUN. The other 3 dogs died within 7 to 11 days, 2 with uremia and one for inapparent reason.

In all the foregoing series in which DEAE-dextran was used in a concentration of 1.0 mg. per milliliter, it was noted that the kidneys were initially revascularized unevenly. There were multiple islands of cyanosis which often required an hour or more to disappear after restoration of the blood supply. Within surface vessels gross aggregates of red cells were visible. These were never seen when only RNA or lactated Ringer's solution was used.

A similar red cell aggregation was noted when 1.0 mg. per milliliter DEAE-dextran

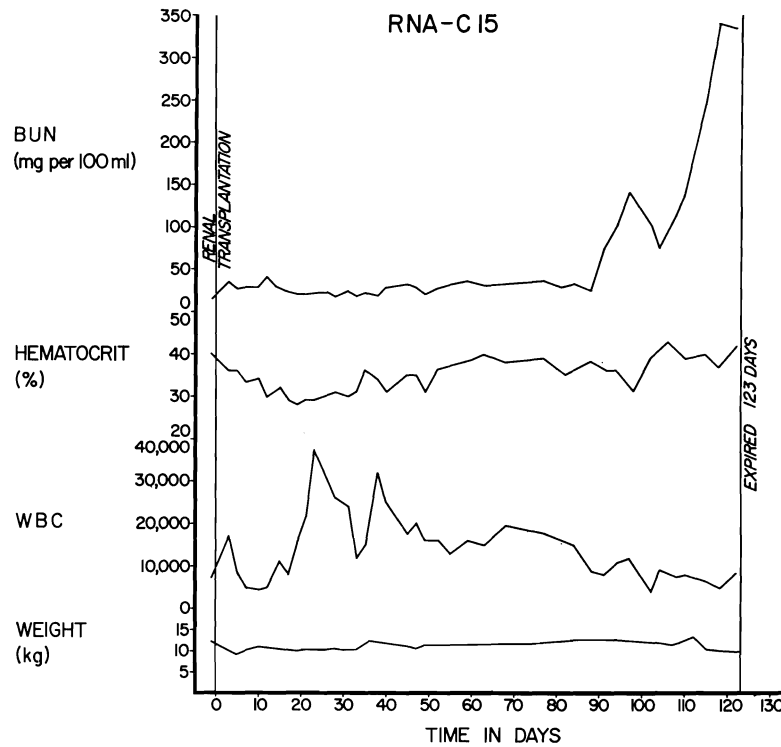


Fig. 2. The course in a dog that lived 123 days after receipt of a renal homograft perfused with homologous RNA plus DEAE-dextran. The final uremia was due to obstruction of the ureteronecystostomy by a calculus. The homograft showed no histologic evidence of rejection.

Table I. Survival in recipients of renal homografts

Group	No. of dogs	Survival (days)			No. of dogs surviving				No. of dogs with nonrejected homografts	
		Mean*	S.D.	Range	15 days	20 days	30 days	40 days		
<i>Test group</i>										
1. Autologous RNA	10	17.8	13.8	9- 55	5	2	1	1	1	
2A. Autologous RNA plus DEAE-dextran	10	18.6	11.5	11- 47	4	3	1	1	1	
3. Homologous RNA	10	17.6	12.5	5- 40	4	3	3	1	2	
4. Homologous RNA plus DEAE-dextran	10	24.2	21.1	9-123	4	3	3	2	3	
				Total	17	11	8	5	7	
<i>Control groups</i>										
5. Homologous RNA plus RNase	10	12.6	4.0	8- 22	2	1	0	0	0	
6. DEAE-dextran	10	13.1	11.3	6- 44	2	1	1	1	0	
7. Lactated Ringer's solution	10	13.4	7.8	7- 35	1	1	1	0	0	
				Total	5	3	2	1	0	

\*For computation of means the maximum credit for individual dogs was limited to 70 days.

was used in the perfusate for hepatic homografts. Two such livers developed fatal acute outflow block. Consequently, the dose of DEAE-dextran per milliliter of RNA perfusate was reduced to 0.3 mg. per milliliter. Seven livers perfused with this modification and transplanted as orthotopic homografts supported life for 46, 14, 13, 11, 7, 7, and 7 days. The average was  $15.0 \pm 14.0$  (S.D.) days. Specific control experiments were not performed but in a recent series of 15 untreated dogs in our laboratory the mean survival was  $8.0 \pm 1.7$  (S.D.) days with a range of 6 to 11 days. To our knowledge, the longest survival ever obtained after orthotopic liver transplantation to an unmodified canine recipient was 31 days.<sup>12</sup>

**Pathologic studies.** The 30 renal homografts in the 3 groups of control animals were all rejected (Table I). The evidence for rejection was dense infiltration of the interstitium by mononuclear cells, focal fibrinoid necrosis of the walls of the arterioles and interlobular arteries, foci of cortical necrosis, and scattered interstitial hemorrhages. An average of 38 percent of the infiltrating cells had pyroninophilic cytoplasm. In one animal that lived for 44 days (Group 6), there were in addition P.A.S.-positive deposits on the glomerular capillary basement membranes. All the homografts were swollen because of interstitial edema.

Seven of the 40 renal homografts that had been perfused with RNA showed no evidence of rejection (Table I). The other 33 kidneys were rejected and did not differ from the controls. Two of the 7 animals not dying of rejection died from uremia 11 and 123 days after transplantation. In the first case the kidney was in the healing phase following acute tubular necrosis presumably inflicted at the time of transplantation. In the other dog calculi formed in the homograft, obstructed the ureter at the ureteroneocystostomy, and led to hydronephrosis and uremia. The remaining 5 homografts which had been retrieved 11 to 55 days after transplantation from dogs dying of pneumonia and wasting were normal.

There was nothing suggestive of rejection

in the 12 renal autografts treated with homologous RNA (Group 8). Autografts from the 2 dogs that died of uremia 7 and 11 days posttransplantation were infarcted though the renal artery and vein had appeared patent at autopsy.

Of the 7 liver homografts perfused with autologous RNA plus DEAE-dextran, all showed evidence of rejection with dense mononuclear cell infiltration around the portal and central veins and necrosis of the centrilobular and midzonal hepatocytes.

## DISCUSSION

RNA has been used in 2 ways in attempts to modify homograft rejection. It has been administered to recipient animals, as first reported by Ashley and associates<sup>2</sup> who claimed a resultant prolongation of skin graft survival. Their observations have been confirmed by two groups of investigators<sup>11, 13</sup> and denied by a third.<sup>15</sup> In Ashley's study and those of Lowe and Axelrod,<sup>13</sup> there was potentiation of homograft survival only if donor-specific RNA was used, although Jolley, Hinshaw, and Peterson<sup>11</sup> claimed the same effect for nonspecific homologous RNA.

Alternatively, RNA has been used by Jolley and Hinshaw<sup>10</sup> to locally treat skin homografts prior to their transplantation. In their experiments, skin was obtained from human cadaver donors, soaked in heterologous (calf liver) soluble RNA, and transferred to the granulating wounds of burned patients. The homografts apparently survived for 5 weeks to 21 months.

The results in the present canine study are in general accord with those reported by Jolley and Hinshaw. A shorter period of perfusion with an RNA extract resulted in the prolongation of function of a moderate number of transplanted kidneys and livers. The effect was not increased by the addition to the perfusate of DEAE-dextran, a presumed RNase inhibitor,<sup>20</sup> but it was abolished by the addition of commercial RNase.

In such experiments the reason is not known for the apparent privilege endowed upon homografts by preliminary treatment with RNA. There is abundant evidence that

fundamental alterations can be induced in mammalian cells briefly exposed to exogenous RNA and that some of them can persist for some time. Such changes, which have been ascribed to the introduction of new genetic information by the messenger fraction of the RNA, can be broadly grouped in those of function<sup>1, 6, 16-19, 24</sup> or structure.<sup>7, 19, 23</sup> It might be reasoned that the messenger RNA known to be present in the extract used in our experiments<sup>14</sup> could have directed a change in the antigenicity of the homograft cells.

Against this explanation was the fact that rejection was delayed as effectively by organ treatment with homologous as with recipient-specific (autologous) RNA. Furthermore, if the increased graft survival were due to induced change in the genetic characteristics of the homograft cells, it would be expected that autografts treated with homologous RNA would undergo at least temporary rejection. This has been said to occur in skin grafts in two reports,<sup>5, 8</sup> a finding which could not be confirmed by Askonas and associates<sup>3</sup> or in the present study. Jolley and Hinshaw<sup>10</sup> similarly concluded that the protective effect of RNA on homografts was by another mechanism, possibly involving a change in cell membrane permeability.

Further work will be necessary to determine if these results have practical implication in clinical transplantation. The increase in homograft survival was limited, but 7 of the 40 renal homografts never developed evidence of rejection. When rejection occurred, however, it was quite severe and indistinguishable from that in the untreated animal. Thus, if RNA conditioning of homografts proves to be useful, the benefit can be expected to be a limited one. It will be interesting to determine whether or not graft conditioning can be advantageously combined with effective host immunosuppression.

### SUMMARY

Canine renal and hepatic homografts were perfused prior to transplantation with RNA prepared from the spleens of the prospective recipients or other indifferent dogs. After

conditioning with either recipient-specific (autologous) or indifferent (homologous) RNA, about a fourth of the homografts subsequently had prolonged function. Maximum survival for the recipients of the kidneys and livers was 123 and 46 days, respectively. The effect was not increased by the addition of a supposed RNase inhibitor, DEAE-dextran, but it was abolished by the addition of commercial RNase. The treatment of renal autografts with homologous RNA did not result in their rejection. The latter finding and the fact that the results after homotransplantation were equivalent with either homologous or autologous RNA suggest that the homograft protection was not due to RNA-induced changes in the genetic characteristics of the cells.

We wish to thank Mrs. M. Berenbein, B.S., for excellent technical assistance.

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## DISCUSSION

**Dr. Joseph E. Murray** (Boston, Mass.). The principle of altering the donor rather than the recipient is instinctively appealing because the possibility of ex vivo pretransplant therapy to the donor organ adds no morbidity to the recipient and may diminish the need for genetic matching of donor and recipient.

The possible mechanisms of altering the donor organ are to change the genetic structure, as mentioned by Dr. Groth, to block release of the antigen, or to cover the antibody receptor sites. The experiments of Dr. Groth and his co-workers used such a short duration and low-flow perfusion that it is promising that any beneficial effect was produced. The nonspecificity of the result is not surprising.

The mechanism of action in these experiments might be delayed recognition of the antigen by the host lymphocytes as they circulate through the graft because, as Strober and Gowans showed clearly, antigen is recognized and processed via lymphocytes traversing the kidney. Possibly the pretransplant RNA therapy covered antigen recognition areas in such a way as to make them less vulnerable to the lymphocyte.

One previous experiment of ours may be pertinent. When long-functioning renal allografts in dogs are retransplanted into their original hosts, they are not rejected. Certainly, long residence in allogeneic hosts, in many instances beyond a year, affords ample opportunity for some genetic change to occur in the graft. Nevertheless, in these experiments no antigenicity was added by such prolonged perfusion.

Finally, the concept of "adaptation" by means of blocking antibodies are promising problems in current clinical organ transplantation. As we well know, most kidney transplants which are destined to fail do so within the first 3 months following transplantation. It is during these 3 months that the host-donor readjustment occurs, "adaptation," if we wish to use the term. This is most likely mediated by the formation of protective or blocking antibodies. Perhaps the late survival is by a secondary mechanism, a final common pathway, quite unrelated to the particular immunosuppressive regimens used at the beginning.

These two papers of Dr. Groth and Dr. Rapaport suggest two ways to minimize the hard core of early transplant failures; with a slight boost, either by antigen pretreatment of the host or by *ex vivo* RNA perfusion of the donor, the grafted organ might be shepherded through that difficult honeymoon of the first 3 months and lead to a compatible permanent marriage.

**Dr. Ronald A. Malt** (Boston, Mass.). The control groups that Dr. Groth and his colleagues used and the tests of statistical significance leave no doubt about the fact that perfusion with RNA increases the length of time the transplanted kidney functions and delays or prevents rejection.

I should therefore like to confine my remarks to asking the group whether they have had time to work on the mechanism for this particular phenomenon, so that the active principle can be exploited.

First of all, is it possible that it is a nonspecific effect of the RNA macromolecules being adsorbed on the surface of the kidney cells? This could easily be proved by trying to wash the kidneys afterward or by the use of other macromolecular substances.

Second, is it possible that the RNA extracted by this technique—which is principally ribosomal RNA and somewhat degraded—entered into the cells, and although it did not change the information capacity of the cells, decoyed the protein synthesizing mechanism into trying to synthesize false proteins on nonsense RNA templated?

Finally, I should like to question the role of ribonuclease. We know that ribonuclease will penetrate from the outside of kidney cells to the inside. In one of the control groups in which ribonuclease was employed, the standard deviation was much smaller than in any other group, indicating that there may have been some consistent effect of ribonuclease in causing kidney rejection. Furthermore, we know from attempts to prepare kidney RNA that lactated Ringer's solution activates ribonuclease in the kidney, and the only way we can,

in fact, prepare electrophoretically undergraded RNA from kidneys is by adding a large amount of RNA to the mixture. So I ask, finally, whether the RNA could have been acting as a sponge to sop up a lot of ribonuclease otherwise activated by perfusion and ischemia in the donor kidney.

**Dr. John A. Mannick** (Boston, Mass.). Several years ago we attempted to accomplish the same end by a slightly different system; namely, attempting to suppress the reactivity of sensitized lymphocytes by exposure to RNA from the animal whose tissues were used to sensitize the cells.

We came to conclusions similar to those of Dr. Groth; namely, that this suppressive effect of RNA was nonspecific. In other words, we had no evidence that we had altered the genetic or epigenetic character of these cells by exposure to RNA.

We felt that perhaps the best explanation was some kind of cell-coating phenomenon.

We found one interesting sidelight observation, and that was that liver RNA, as opposed to lymphoid RNA, was totally ineffective, and I wonder if Dr. Groth has tried RNA extracted from other than a lymphoid organ in his experiments.

**Dr. Groth** (closing). The interesting suggestions concerning the underlying mechanism by Dr. Murray and the very intriguing suggestions that were made by Dr. Malt are fascinating to me. However, we have so far no data that can explain why RNA prolongs homograft survival, and I think we have to conclude for the moment that the mode of action of the RNA in this kind of experiment is unknown. We would certainly like to explore this further.

We have not used anything but RNA extracted from spleens. We intend to try unspecific heterologous RNA, RNA that you can buy from any chemical company. If that would work, this would certainly make things easier. The phenol extraction takes some time.