Transplantation of Canine Larynx and Its Immunology

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INTRODUCTION

Although the larynx is not vital organ, transplantation of this structure has been under investigation in recent decade.[1)2)] The aim of larynx transplantation is to restore normal functions of the larynx following laryngectomy. These functions involve a normal airway, control of the airway during deglutition and phonation. Therapy for cancer or injury of the larynx requires total removal of this structure in certain instances. Following laryngectomy with tracheostomy the airway remains to be safe, but uncomfort complications, such as crusting and bleeding from the tracheostomy occurs. With regard to phonation, some patients learn esophageal speech, others use mechanical sound, or reconstruction surgery is necessary.

The first application of the larynx transplantation to human was performed in 1969.[3)] However, the widespread adoption of this procedure as a remedy seems to be too early because of several obstacles to obtaining effective and harmless transplantation. Immunological events, which threaten the transplanted organ, are the chief obstacle. Problems of the reinnervation of laryngeal nerve and sound production are also important factors in successful transplantation of the larynx.

ANATOMIC CONSIDERATION OF THE CANINE LARYNX

Most investigators have used dogs as animal model because the size and shape of the larynx between human and dog is similar. The difference of arterial supply between canine and human larynges is noted in Figure 1. The canine larynx is supplied by four arteries which are derived from the common carotid artery. While

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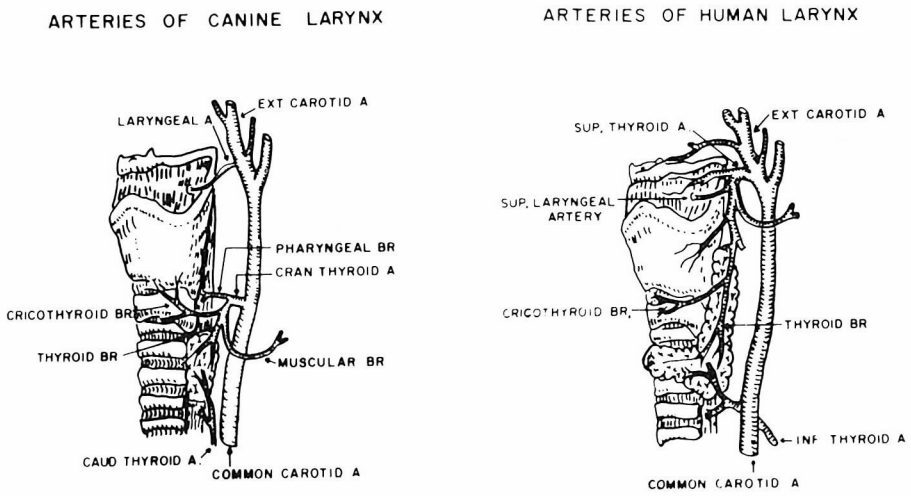

Figure 1. Arterial Supply of the Canine and Human Larynges

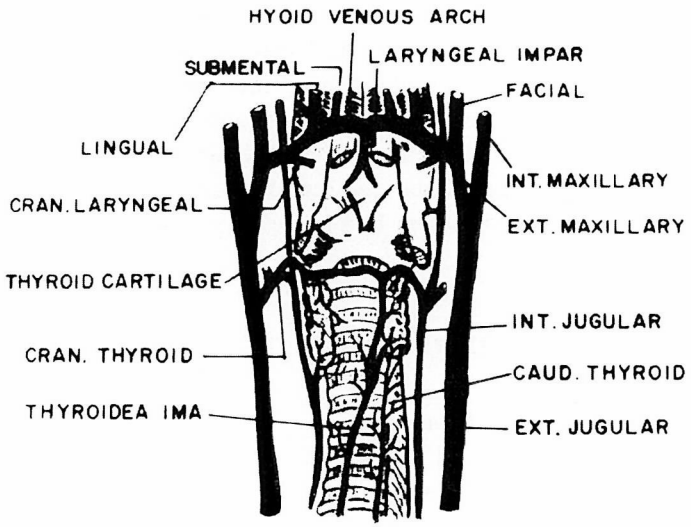

Figure 2. Venous Drainage of Canine Larynx

human larynx receive the main blood supply from the superior laryngeal artery, the cranial thyroid artery arising from the common carotid is the main vessel to the canine larynx. The venous drainage of the canine larynx is illustrated in Figure 2. The venous return is mostly through the cranial laryngeal vein and the laryngeal impar. These two veins joint to the lingual vein which forms the external maxillary vein by conjunction with the facial vein. The nervous supply of the canine larynx is derived from branches of the vagus. According to Ogura et al[1] the diameter of common carotid arteries in 13 to 25 kg body weighed dogs is between 3 and 4 mm, whereas that of the cranial thyroid artery is around 2 mm.

PROCEDURE

After the intravenous anesthesia with sodium pentbarbital the supine position was made, stretching the neck. A midline incision was made and the larynx isolated. During the isolation of the larynx special attention was given to the preparation of vessels to avoid their injury. Since organ transplantation requires the immediate reconstitution of adequate blood supply, the anastomosis of arteries supplying blood to the larynx must be performed. Ogura et al[1] found that adequate blood flow to the canine larynx is provided by retaining only the cranial thyroid arteries. Therefore, four types of anastomotic sites for the canine larynx could be designed as shown in Figure 3. The TT-type indicates the anastomosis at the site of both cranial thyroid arteries, and the CT-type involves the anastomosis of one side cranial artery and of the common carotid artery of the opposite side. In the CC-type the anastomosis is done at the site of both common carotid arteries, while in the C-Type the anastomosis is at the common carotid artery of only one side. However, the cranial thyroid artery is so small in diameter that the anastomosis is carried out smoothly. At the present time, it is well recognized as the easiest and most reliable way to rejoin vessels that a section of the common carotid artery, with the attached cranial thyroid artery, is removed and reanastomosed. In our study the CC-Type was used principally. Bilateral facial and external maxillary veins were chosen for anastomotic sites of veins. The vessel anastomosis can be accomplished by use of the Nakayama eyelet-rivet clamp. Neurorrhaphy of recurrent nerves was performed.

RESULTS

Results of our study indicated that in cases treated with no immunosuppressive regimens the transplanted larynx was rejected within 8 days and or became necrotic.

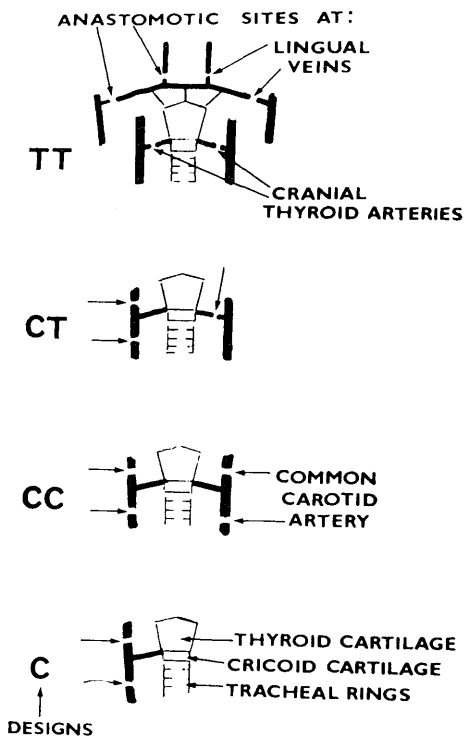

Figure 3. Schematic Drawing Showing the Model Designs of the Anastomatic Sites for the Canine Larynx

Insufficiency of the anastomosis, acute rejection and infection could be considered as the cause of the necrosis of the transplanted larynges.

GRAFT REJECTION

The rejection or acceptance of transplants between individuals of the same spacies is governed by histocompatibility antigens. The specificities of transplantation antigens (histocompatibility antigens) are under the control of genes at the large number of loci. The transplantation antigens have been extensively investigated in mice and human but not in dog. When transplantation is done between individuals who have same transplantation antigens, no rejection phenomena occur. However, large differences in transplantation antigens evoke strong rejection reactions. As shown in Figure 4 the recognition of foreign transplantation anti-

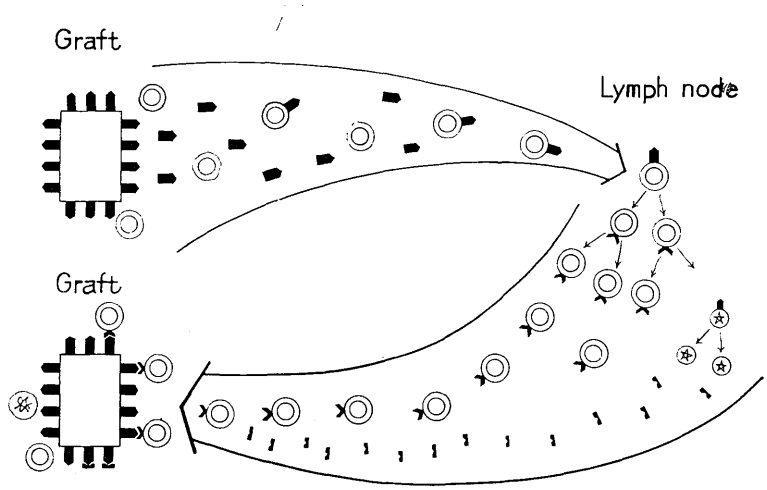

Figure 4. The Mechanism of Allograft Rejection

Tissue antigen of t he donor

Immunologically competent cell of the recipient

Sensitized lymphocyte

Antibody

Antigens of donor's tissue are recognized by immunologically competent cells of the recipient. This recognition responds with both sensitized lymphocytes and antibody formation which react with antigens of donor's tissue. Subsequently, mononuclear cells accumulate, and the graft destruction becomes evident.

gens by immunological competent cells of the host initiates the graft rejection. Immunological competent cells that recognized antigens divide into many sensitized lymphocytes in regional lymph nodes. Afterwards a number of sensitized lymphocytes infiltrates to vessels in the graft. Humoral antibodies are also produced. Sensitized lymphocytes and humoral antibodies accumulate to the graft, triggering an inflammatory process which further progresses in the graft necrosis.

We are now investigating characteristics of morphologic changes in graft rejection of the larynx transplantation.

IMMUNOSUPPRESSION

There are four approaches to control the graft rejection: these are selection of a donor and recipient with least possible antigenic differences, nonspecific immunosuppressive regimens, antilymphocyte serum (ALS), and immunologic tolerance (Table 1).

TABLE I IMMUNOSUPPRESSIVE REGIMENS

Tissue Typing or Histocompatibility Test
Nonspecific Immunosuppressive Methods
Antilymphocyte Serum (ALS)
Immunological Tolerance

The selection of donor and recipient is known as tissue typing or histocompatibility testing and the usefulness of matching donor and recipient by this method has been proven by long survival of properly typed grafts. However, this procedure is not simple in human and has yet to be developed in dog.

Various immunosuppressive regimens have been attempted. Among those reported are irradiation, surgical methods including thymectomy, splenectomy, and thoracic duct fistula, and chemicals, such as 6-mercaptopurine, azathioprine (Imuran), steroid, actinomycin, and methotrexate. However, many of these forms of therapy are associated with undesirable side effects and, therefore, a more effective and less toxic form of immunosuppressive treatment was desirable.

Since 1963, when Woodruff and Anderson[4] reported prolonging skin allograft survival in rats by use of heterologous antilymphocyte serum (ALS), this substance has come to the forefront as a promising new form of immunosuppressive therapy. We have studied properties of horse anti-dog thymocyte plasma with the intention of using it to promote the survival of the transplanted canine larynx.

ALS was made by immunizing horses with dog thymocytes. The thymus was excised from puppies, cut into small fragments, and injected into horses. Afterwards blood was drawn from immunized horses and the plasma was separated and incuvated in a water bath to eliminate complement. This antiplasma was absorbed with dog red cells in order to remove antibodies against dog red cells. Immunoglobulins were fractionated from the antiplasma and were then injected into experimental dogs (Figure 5).

We prepared ALS by two different immunization methods. One of them involved repeated injections of the antigen over a long period of time to obtain higher in vitro activities for cytotoxicity and leukoagglutination (Figure 6). The other regimen is characterized by two injections over a short period of time

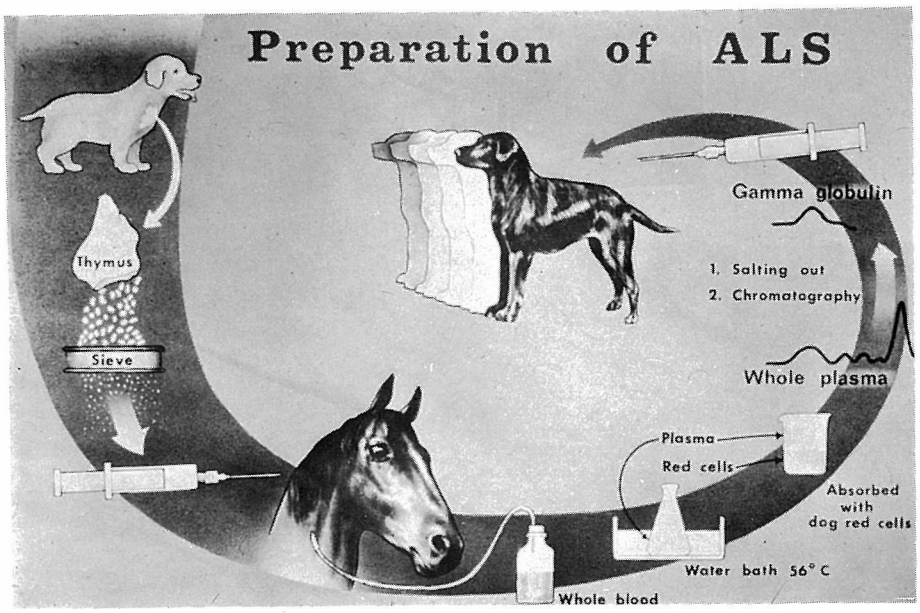

Figure 5. Preparation of ALS

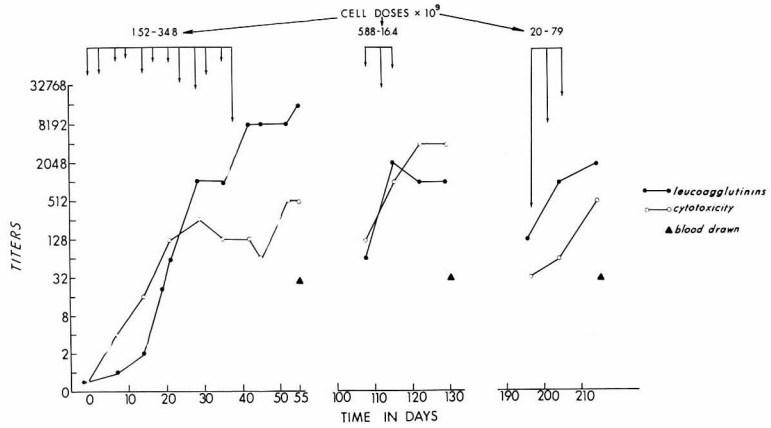

Figure 6. Cytotoxic and Leukoagglutinin Responses in a Horse Immunized by Multiple Injections of Thymocytes

(Figure 7). We obtained two different batches of ALS and their in vitro titers of cytotoxicity and leukoagglutination are illustrated in Table 2.

The administration of ALS causes immune reactions and formation of antibodies by the recipient to proteins contained in ALS and this presents a likely cause of many dangerous side effects. Therefore, fractionation of ALS offers the advantages of permiting the administration of large doses of effective antibody in small amounts

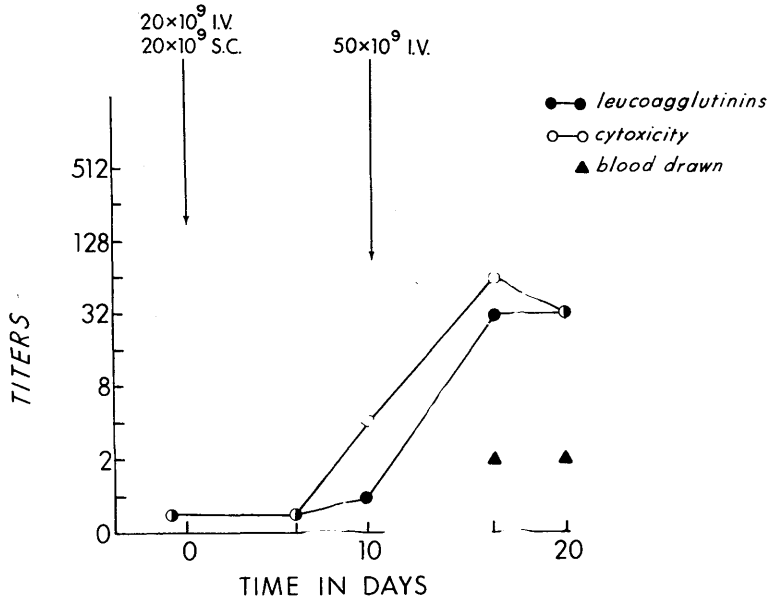

Figure 7. Cytotoxic and Leukoagglutinin Responses in a Horse Immunized by the Two-pulse Method

TABLE II IN VITRO TITERS OF TWO DIFFERENT BATCHES OF ALS

	Cytotoxicity	Leukoagglutination
High titer-ALS	1 : 512 – 1 : 4096	1 : 512 – 1 : 16384
Low titer-ALS	1 : 32 – 1 : 64	1 : 32

and avoiding the injection of large amounts of antigenic non-antibody proteins. Salting out with ammonium sulfate was adopted as a first step for the purification of antibodies. The crude gamma globulin (CGG) obtained by salting out was further fractionated by G-200 Sephadex gel filtration and by DEAE-cellulose column chromatography (Figure 8). Column chromatography is probably the most advantageous method for use in the preparation of immunoglobulin G (IgG) of the highest purity. However, this method is not suitable for mass production of IgG. On the other hand, acceptably pure IgG may be prepared from relatively large volumes of serum by use of the batch separation method. This method also lends simplicity and rapidity to the process. Therefore, we fractionated IgG from both the high and low titer ALS by use of batch production method using DEAE-cellulose.

Figure 9 exhibits the distribution of in vitro activities in each immunoglobulin. Cytotoxicity was present in 7S or IgG, leukoagglutinin activity resided both in 7S and 19S, and hemagglutination existed mainly in 19S.

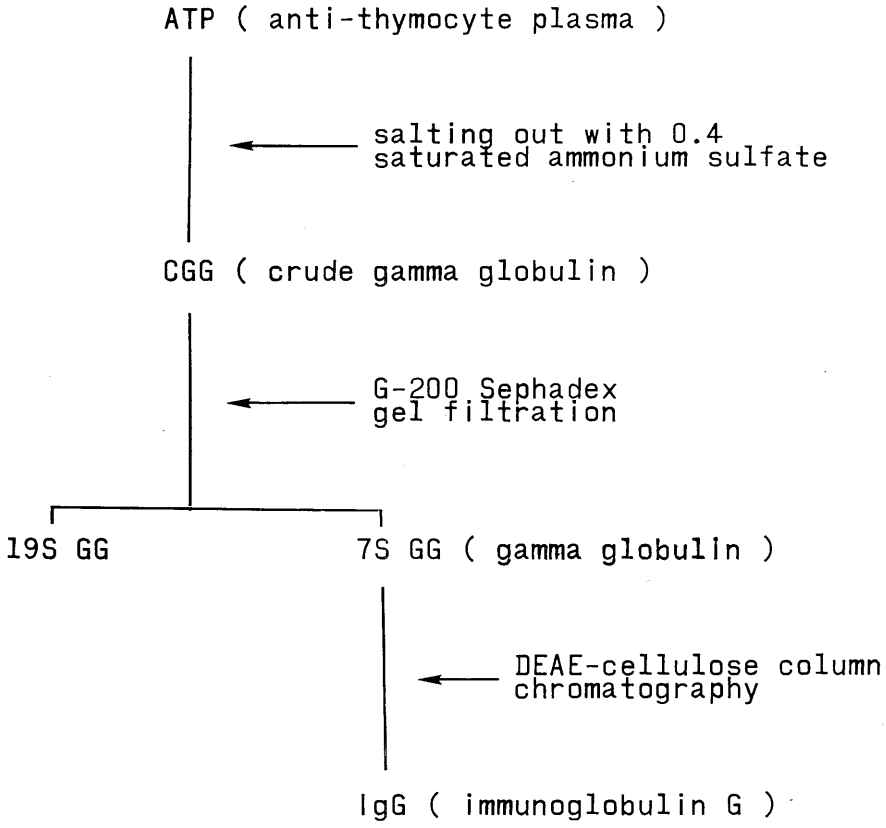

Figure 8. Purification of Immunoglobulins from ALS

The CGG or 7S gamma globulin derived from both the high and low titer ALS was injected into dogs and changes of total WBC and small lymphocyte count were observed. The dosage of the immunoglobulin injected was 10 mg/kg of body weight and it was given subutaneously or intravenously. As shown in Figure 10 absolute small lymphocyte counts decrease quickly after the injection of both the high and low titer immunoglobulins. However, the degree of the lymphopenia was not correlated with the in vitro titers of the immunoglobulins for cytotoxicity and leukoagglutination. Five dogs were inoculated daily with the

		Cytotoxicity	Leukoagglutination	Hemagglutination
High titer	7S	+++	++	++
	19S	++	+++	++++
	IgG	+++	+++	+
Low titer	7S	+	++	+
	19S	−	++	+++
	IgG	+	++	+

Fig. 9. Distribution of In Vitro Activities

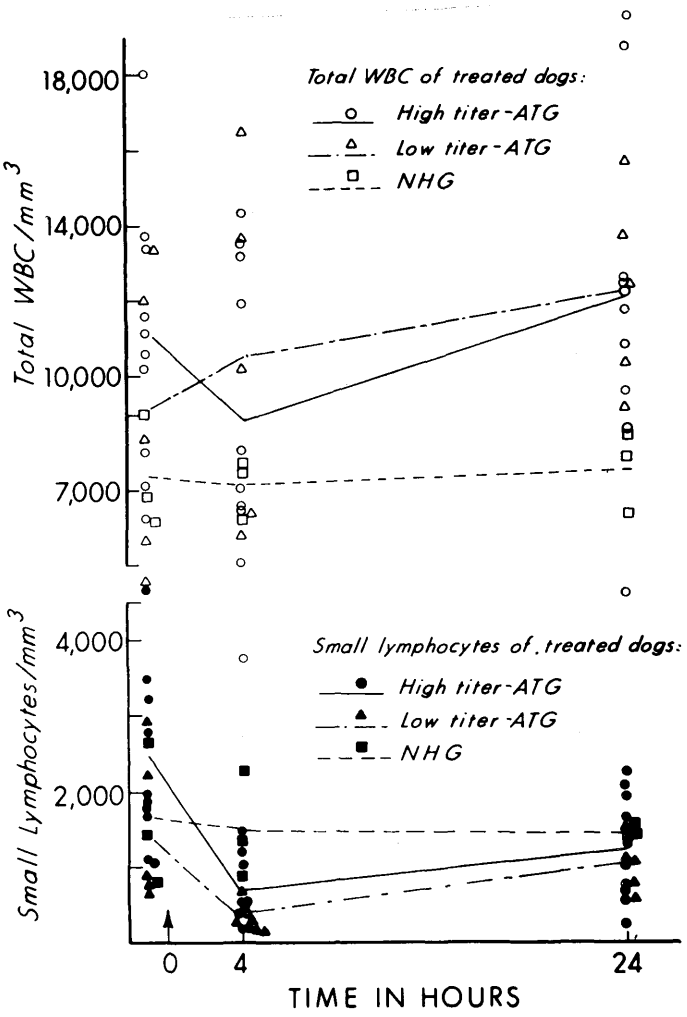

Figure 10. Changes in WBC and Small Lymphocyte Counts of Dogs Following a Single Injection of ALS and NHG.

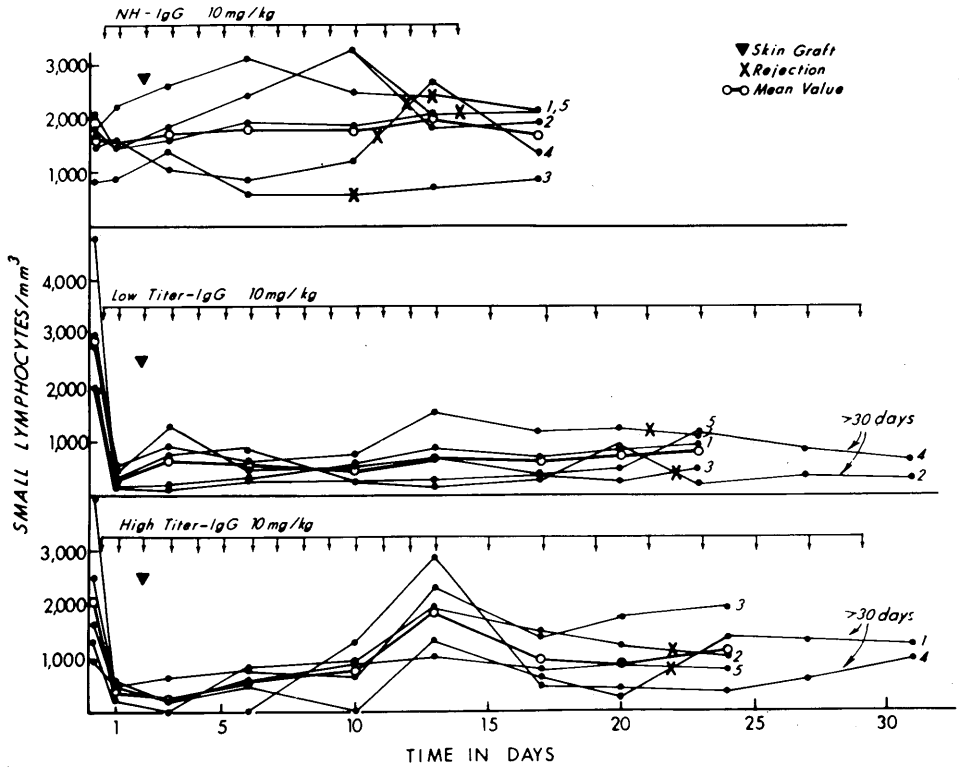

Figure 11. Changes of Peripheral Blood Small Lymphocytes Following Injections of ALS-IgG

high titer immunoglobulin until the skin graft was rejected. A lymphopenia of less than 1000/mm³ was maintained during the injections regardless of skin allograft rejection. Serial injections of the low titer immunoglobulin were given to four dogs. A lymphopenia was observed. Changes in peripheral blood small lymphocyte counts were observed during the course of serial injections with the high titer-IgG, low titer-IgG, and NH-IgG (Normal horse-IgG). The results are illustrated in Figure 11. Both in dogs given the high titer-IgG and those given the low titer-IgG, an absolute small lymphocyte counts decreased quickly after the initial injection and remained depleted to less than pre-injection values during the course of injections. Dogs treated with NH-IgG as a control, no remarkable changes in lymphocyte counts were detected. Two dogs were injected with the high or low titer 19S gamma globulin, respectively. But these injections did not cause such a profound fall in the small lymphocyte count in the peripheral blood as did the high and low titer 7 S immunoglobulins. These injections of ALS-

TABLE III SURVIVAL OF SKIN ALLOGRAFTS IN DOGS TREATED WITH DIFFERENT GAMMA GLOBULIN FRACTIONS OF THE HIGH AND LOW TITER ANTITHYMOCYTE (ATP)

Treatment		No. of dog	Survival of skin allografts (days)
No treatment		3	7, 8 and 13
NHG		2	7 and 8
High titer ATP	—CGG	2	10 and 21
	—7S	3	17, 24 and 25
	—19S	1	9
Low titer ATP	—CGG	2	17 and 22
	—7S	2	19 and over 60
	—19S	1	13

Key: NHG: Normal horse crude gamma globulin
7S: 7S gamma globulin
19S: 19S gamma globulin

TABLE IV THE EFFECT OF ATP-IgG ON SKIN ALLOGRAFT SURVIVAL

Group	Treatment	Number of Cases	Survival Days of the Skin Allograft
A	High titer-IgG	5	20×2, 21,>50×2
B	Low titer-IgG	5	18, 19, 20, 45,>50
C	NH-IgG	5	8, 9, 10, 11, 12
D	No treatment	5	8, 10, 11×2, 13

Key: NH-IgG Normal horse IgG
×2, 2 cases
>50, over 50 days

immunoglobulins were not directly responsible for any deaths. But one dog developed an abscess at the injection site and another one dog had systemic reactions such as hypersalivation, hyperventilation, and vomitting.

During the course of injections each dog was given an allograft for testing the efficacy of these ALS-derivatives to prolong allograft survival. Table 3 and 4 show the result. These data demonstrate that the activity promoting the prolongation of the skin allograft survival resided in the 7 S gamma globulin or IgG obtained from both the high and low titer ALS. Although only a few cases were tested on the efficacy of the 19 S gamma globulin, because fractionation of 19 S gamma globulin has not yet been solved satisfactorily for mass production, the 19 S of both the high and low titer ALS did not enhance the skin graft survival.

Antibody formation against the injected ALS-immunoglobulin was investigated using immunodiffusion techniques. Precipitation antibodies were found in sera taken two or three weeks after commencing injections. This occured before the skin grafts were rejected. From these results it should be considered that antibodies to ALS-immunoglobulins not only might cause harmful side effects, such as anaphylaxis, and kidney damage, but also might interfere with the activity of ALS, resulting in poor allograft survival.

Although ALS is a potent immunosuppressive agent, it is still less than a perfect guaranty of the viability of transplants for an extended or indefinite period of time, according to many investigators[5)6)7)] as well as us. So various combined treatments of ALS with other immunosuppressive regimens have been attempted for the purpose of increasing the immunosuppressive effects of ALS. On the other hand, it has been elucidated that the thymus plays an important roles in immune responses. Among immunologic functions of the thymus, it is noteworthy that the recovery of immune responses after treatment with an immunosuppressant is thymus dependent. Therefore, it could be considered that thymectomy in ALS-treated animals, even in the adult, retards the recruitment of immunologically competent lymphocytes, thus to enhancing the effect of ALS for prolonging allograft survival. We tried the use of ALS-IgG in adult thymectomized dogs and obtained results described as follows: 1. Adult thymectomy in dogs caused a slight depletion of peripheral blood small lymphocyte count, but no effect on the survival of allografts of skin was observed and 2. adult thymectomy previous to administration of ALS-IgG did not augment the immunosuppressive effect of these ALS-IgG.

The induction of specifically immunologic tolerance to the donor antigens responsible for the rejection has the greatest promise, theoretically. However, the method is practically the least well developed.

REINNERVATION OF LARYNGEAL NERVES

The reinnervation of laryngeal nerves is another important problem in successful transplantation of the larynx. The importance of reinnervation of the larynx arises from two interdependent factors: 1. motor control of the intrinsic muscles of the larynx is necessary if the transplanted organ is to perform its airway protection and phonatory functions, and 2. normal sensation is needed for proper functioning of the larynx during conscious and reflex activity. Although reinnervation of the transplanted larynx depends ultimately on proper control of rejection, failure to obtain good function might be attributed to several causes, such as misdirection of the regenerating nerve fibers and reduction in the number of motor units. To accelate the regeneration of the laryngeal nerves, U-9189 (1, 1, 3-tricyano-2-

amino-1-propene) was administered to dogs with paralyzed larynx made by cutting the recurrent nerves the effects of this drug on the reinnervation of the laryngeal nerve were demonstrated.[8)]

CONCLUSION

Even though the goal of the larynx transplantation is still away in our hands, we shall keep the endeavor to solve problems disturbing the successful transplantation of the larynx.

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