Immunologic Competence and Induction of Neoplasms by Polyoma Virus. (30311)

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The development of immunologic competence is impaired in most strains of mice subjected to neonatal thymectomy. This is not an absolute impairment since the capacity to respond to some antigens appears unaffected(1,2). The ability to reject allogeneic grafts of normal or neoplastic tissues however appears to be generally influenced and detectable especially when antigenic differences are slight, for example, if donor and recipient share the same histocompatibility-2 (H-2) gene. In some strains this impairment of homograft rejection is observed even in mice thymectomized as late as 6 days, and in one strain combination as late as 30 days(3).

The animal with a deficient immune mechanism is therefore useful for a study of host resistance to spontaneous and experimentally induced neoplasms, particularly to those neoplasms which are known to be antigenic in the syngeneic or autochthonous host(4-9) and which would be expected to initiate a homograft-type reaction. The frequency and progression of neoplasms induced by certain chemical carcinogens(10) and by polyoma virus(11-13) are indeed reported to be influenced by neonatal thymectomy and at least in the case of polyoma-induced neoplasms, the evidence suggests that the immunologic capacity of the host governs susceptibility and resistance to tumor induction (14).

In C57BL/6JN mice thymectomy within 24 hours of birth led to a higher frequency of tumors at many different sites following injection with S-polyoma virus(15). In the highly resistant C57BL/KaLw strain thymectomy performed within 24 hours of birth or later at 3 days led to a striking susceptibility to the oncogenic effects of the LID_1 strain of polyoma virus when virus was injected shortly after thymic ablation(13,14). In C57BL mice thymectomized at 7 days and irradiated at 21 days of age polyoma virus initiated neoplasms when introduced as late as 60 days of age(16); whole body irradiation alone, however, of adult C3H mice has been shown to increase susceptibility to polyoma virus(17). Restoration of 1-day thymectomized mice through the introduction of competent adult spleen cells provided the ability to resist the oncogenic effects of virus (14), in the absence of thymic tissue.

As a continuation of studies of the role of the thymus in tumor induction with polyoma virus we have examined the following: 1) The response to virus of other resistant and sensitive strains of mice thymectomized at 3 days of age. Thymectomy at this time prevents the "wasting" characteristic of the strains employed in this study, 2) The response to polyoma virus of restored 3-day thymectomized C57BL mice. Restoration was accomplished by intravenous injection of dissociated syngeneic adult spleen cells, by subcutaneous thymic grafts or by syngeneic thymic tissue in cell impermeable Millipore diffusion chambers, 3) Study of the effect of neonatal thymectomy on prolongation of the susceptible age to polyoma virus oncogenesis; normal mice older than 2 weeks of age are usually insensitive to the oncogenic influence of polyoma virus, and lastly, 4) A study of polyoma virus growth curves and of antiviral antibody formation in thymectomized C57BL mice and their intact litter mates. The possibility exists among others that differences in virus growth rates in thymectomized *vs* intact mice may account for differences in susceptibility to polyoma virus.

Material and methods. Mice: Inbred C57BL/KaLw, C3Hf/Lw, C3Hf/Bi, and an F_1 hybrid C57BL/KaLw $\times$ C3Hf/Lw, (Bl $\times$ C3H)F_1 were all raised in this laboratory. Polyoma-type neoplasms developing spontaneously within our inbred colonies over the past 10 years have been rare.

Virus: For the most part, a standardized large plaque polyoma virus stock(18) has been used. This was derived from the LID-1 strain. In a limited number of experiments

Dulbecco's small plaque variant, S/M, was used. Both were grown on mouse embryo cells. In mice 20 days or younger 0.05 ml was usually injected subcutaneously while in those recipients older than 20 days 0.1 ml was used; specific details are given in the Tables. These inocula represented concentrations of virus of 2×10^6 and 4×10^6 plaque forming units (PFU).

Thymectomy: Thymectomy or sham-operation were performed on mice at 3 days of age, except when otherwise noted, by a method adapted from Miller(19). Littermate controls (either sham-operated or intact) were maintained for each litter.

Restoration procedures: Dissociated spleen cells from adult 4- to 6-week-old syngeneic donors were prepared according to the method of Billingham(20) except that Hanks' solution was used and the cells were washed 3 times before injection. Each recipient received at 4 days of age, *i.e.*, at 1 day following thymectomy, unless otherwise noted, 6 to 12×10^6 spleen cells inoculated intravenously by way of the orbital branch of the anterior facial vein.

Syngeneic thymic grafts obtained from neonatal donors were implanted in the subcutaneous tissues in the right axillary region. The graft was placed in the implantation site with curved forceps from a ventral incision using anesthetized recipients. Male to male and female to female donors and recipients were used.

The cell-impermeable Millipore® chambers used have been described(21). In the present study a smaller chamber, 10 mm diameter, was used with Millipore membranes of an average pore size of 0.45 μ. An entire thymus from neonatal syngeneic donors was used. The chambers were installed intraperitoneally when the thymectomized recipients were 2 weeks of age.

Histology: Sections of neoplastic and suspected neoplastic tissues as well as lymphoid tissues were routinely fixed in Tellyesniczky's fluid and stained with hematoxylin and eosin. Microscopic sections were taken of the upper mediastinal area when gross observation suggested the presence of a thymic remnant. Those animals incompletely thymectomized are not included in the tabulations.

Virus growth studies and hemagglutination inhibition antibodies: C57BL mice thymectomized at 3 days of age and their intact litter mates were infected with 1×10^5 plaque-forming units (PFU) per mouse of LID-1 virus at day 7. At various times after infection 3 mice from each group were sacrificed and kidneys, livers and salivary glands pooled separately and weights determined. These were suspended in 1 ml buffered saline and kept frozen at —70°C until assay. The tissues were homogenized and cellular debris removed by 3 cycles of low speed centrifugation at $1000 \times g$. The concentrations of virus were assayed in these supernates by the method of Dulbecco and Freeman(18) and expressed as PFU per 0.1 g of tissue.

Hemagglutination-inhibition antibody (HI) titers against the LID-1 strain of polyoma virus were determined in 3-day thymectomized mice and their intact litter mates according to the method of Rowe *et al*(22). Six of each group received 1×10^5 PFU of virus at day 7, and titers were determined at several times from 4 to 30 days later. One gamma (γ) of blood was drawn from the tail of each mouse and diluted 100-fold in buffer. Each mouse was then bled successively. The microtiter technique(23) was used to determine HI antibody titers.

Results. The usual stigmata associated with thymectomy performed within 24 hours of birth were not observed in our 3-day thymectomized mice. Growth curves of thymectomized and intact litter mates were similar. There was little or no evidence of "wasting" and there did not occur in general lymphoid depletion in organs or in peripheral blood as determined in the C57BL and C3H strains. C3H mice rejected AKR skin grafts normally and the mean hemolysin titers at 7 days to sheep erythrocytes were similar to intact litter mates. It is known, however, that thymectomy at this time still has an effect on the development of immunologic reactivity to certain antigenic stimuli(24).

A high frequency of polyoma-type neoplasms principally of the parotid salivary glands was observed in all strains of mice thymectomized at 3 days of age especially if virus was introduced within the first week of life (Table I). In addition, the period of

TABLE I. Polyoma-Induced Neoplasms in Several Strains of Mice Following Early Thymectomy.

Strain	Group	No. litters	Virus strain	Virus conc	Age at virus infection	No. mice tumorous/ No. infected	Latent period Mean (range)
C57BL	Thymect (3 day)	8	LID-1	2×10^6 PFU	4-7 days	18/26 (73%)	2½ (2-4½) mo
	Control					0/15	–
	Thymect (3 ")	15	"	2×10^6 PFU	14-18 "	21/42 (50%)	3 (2½-4) "
	Control					0/39	–
	Thymect (1 ")	10	"	2×10^6 PFU	4-7 "	15/19 (78%)	2 (1½-3) "
	Control					1/37	4½
(B1 × C3H)F_1	Thymect (3 ")	4	"	2×10^6 PFU	10-15 "	3/11 (28%)	3½ (4-5) "
	Control					0/8	–
	Thymect (3 ")	7	"	4×10^6 PFU	16-30 "	4/25 (16%)	5½ (2½-11½) "
	Control					0/15	
	Thymect (1 ")	6	"	2×10^6 PFU	5-6 "	8/10 (80%)	3½ (2-5½) "
	Control					0/16	–
C3Hf/Lw	Thymect (3 ")	5	"	2×10^6 PFU	7-16 "	9/15 (60%)	5½ (4-7½) "
	Control					0/10	
	Thymect (3 ")	7	S/M	1×10^7 PFU	4-13 "	6/21 (29%)	9½ (8½-11½) "
	Control					0/15	–
C3Hf/Bi	Thymect (3 ")	5	LID-1	2×10^6 PFU	8-14 "	16/19*(84%)	3½ (2-12) "
	Control					2/10	5½ (4-7) "

* Multiple neoplasms. See text.

TABLE II. Response of Thymectomized and Restored C57BL Mice to LID-1 Strain of Polyoma Virus.

	Group	No. litters	No. with tumors/ Effective No. mice	Latent period Mean and (range)	Remarks
A	Thymectomized at 3 days		20/31 (65%)	2½ (2-4) mo	LID-1 polyoma virus injected subcutaneously at 13 to 18 days 4 × 10^6 PFU/mouse
	Thymectomy + dissociated spleen cells	21	2/29 (7%)	5, 7 "	
	Sham and intact*		0/42		
B	Thymectomy at 1-3 days		9/24 (38%)	6 (3-12) "	Polyoma virus injected subcutaneously at 20-30 days 4 × 10^6 PFU/mouse
	Thymect + Millipore chambers containing neonatal thymus at 14 days	12	1/ 8 (12.5%)	1½ "	
	Intact		0/20	-	
C	Thymectomy at 1 day		9/12† (75%)	2½ (2-4) "	Polyoma virus given at 14 days 2 × 10^6 PFU/mouse
	Thymectomy + syngeneic thymic graft at 1 day	7	2/12‡ (17%)	3½, 9 "	
	Intact		0/30		

* No differences in response between sham-operated and intact mice; other controls in B and C were therefore not given a sham operation.

† Two mice died of "wasting" before tumor induction.

‡ One animal died "wasted" but with a parotid gland neoplasm at 3½ months of age.

sensitivity to the oncogenic effects of polyoma virus was considerably lengthened, 38% of C57BL mice, for example, developing parotid gland neoplasms when injected with virus as late as 30 days of age (Table II, Fig. 1). Intact mice of most strains older than 2 weeks at the time of polyoma virus injection usually do not develop polyoma-type neoplasms(25). The intact or sham-operated mice used in the present study, except for C3Hf/Bi, were highly resistant to the concentration of LID-1 virus even when introduced early in life.

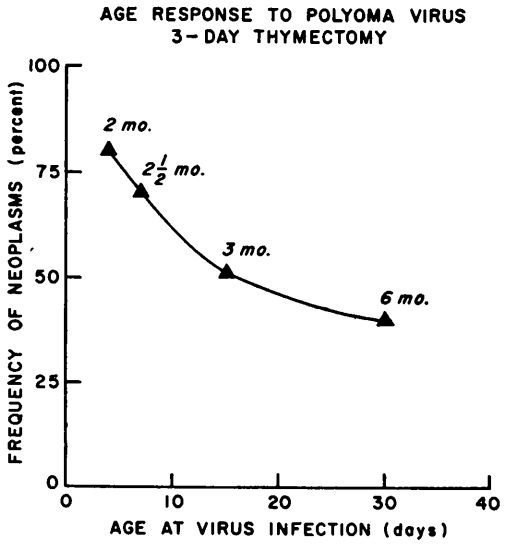

FIG. 1. Frequency of parotid gland neoplasms in C57BL mice thymectomized at 3 days. Polyoma virus infection, 2-4 × 10^6 PFU initiated at time indicated. Average latent periods for neoplasms in months given above each point.

The response to polyoma virus of C57BL and F_1 mice thymectomized within 24 hours of birth is shown for comparison in Table I.

Parotid gland tumors were also observed in thymectomized C3Hf/Lw mice given the relatively non-oncogenic small plaque, S/M, polyoma variant although the latent period was considerably lengthened over that observed in thymectomized mice given the LID-1 strain. Malmgren *et al*(15) have observed an increase in the frequency of polyoma-induced neoplasms in thymectomized mice infected with an "attenuated" strain of virus (M-polyoma).

The highest frequency and multiplicity of polyoma-type tumors was observed in the susceptible C3Hf/Bi strain of mice. Here, although LID-1 virus was given late, at 8-14 days of age, multiple neoplasms were found in most 3-day thymectomized mice. In addition to salivary glands, the sites involved were mammary glands, hair follicles, subcutaneous connective tissue, kidney and bone. In contrast, in thymectomized C57BL strain mice, the neoplasms were for the most part localized to the salivary glands.

Interestingly, the F_1 hybrid obtained by crossing 2 strains C57BL and C3Hf/Lw both resisting LID-1 strain polyoma virus onco-

genesis, appear in this study to be more insensitive than either parental strain except in those F_1 mice thymectomized within 1 day of birth.

Fig. 1 shows that there is a decreasing response of 3-day thymectomized C57BL mice to 2 to 4×10^6 PFU polyoma virus as the animal matures, although there remains a relatively high degree of sensitivity if polyoma virus is given as late as 30 days after thymectomy. This may represent development with age of immunologic competence to the extent of providing some resistance to polyoma virus. This age-response was not tested beyond 30 days of age; however it is suggested from the work of Dalmasso *et al*(26), who studied graft *vs* host reactivity and in our studies of "room infection" with polyoma virus(27), that the early thymectomized animal does not attain a degree of immunologic function characteristic of the intact or sham-operated litter mates.

Immunological defects resulting from early thymectomy, lymphoid cell depletion and failure of growth in those strains of mice which show "wasting" all can be prevented by several methods: 1) grafting of newborn thymic tissue, 2) administration of adult lymphoid cells from syngeneic donors, or 3) intraperitoneal installation of cell-tight Millipore chambers containing neonatal thymic tissue (21,28). It will be seen in Table II that all 3 methods of restoration prevented the induction by polyoma virus of the usual polyoma-type neoplasms encountered in thymectomized mice. In group A, splenic cells from adult syngeneic donors were effective whether given intravenously, 6 to 12×10^6 dissociated cells at 4 days of age or intraperitoneally, 3×10^7 cells at 7 days and again at 9 days of age. The results were similar in the 2 groups and are combined in the Table.

Millipore® chambers were recovered at 12 months from the restored C57BL mice in Group B. In all, the mass of existing tissue was principally necrotic but viable tissue was observed in the form of fibroblasts and nests of epithelial-reticular cells. Thymic tissue in Millipore chambers was as effective as neonatal syngeneic thymic grafts in restoring the ability of C57BL mice to resist induction of tumors by polyoma virus. Fig. 2 shows the

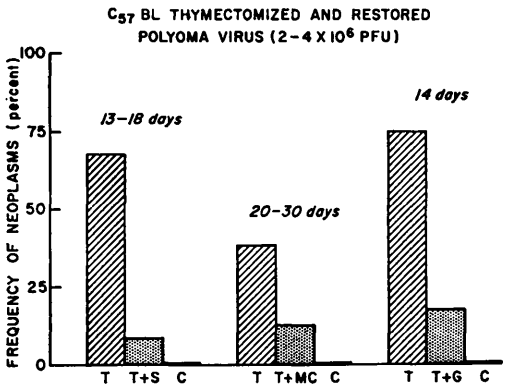

FIG. 2. Frequency of parotid gland neoplasms in the 3 groups of 3-day thymectomized C57BL mice. T = thymectomy, C = intact, T + S = thymectomized mice restored with adult spleen cells, T + MC = thymectomized mice bearing Millipore diffusion chambers with thymic tissue, T + G = thymectomized mice bearing syngeneic thymic grafts. Age in days at which LID-1 polyoma virus was injected is given above each group.

relative effects of restoration by the 3 methods employed.

The effectiveness of adult spleen cells from syngeneic donors in restoring the potentiality to resist polyoma virus oncogenesis was more striking in these studies than previously found, employing 1-day thymectomized C57-BL mice(14). In the present experiments virus was injected from 9 to 14 days after introduction of dissociated spleen cells whereas previously only 2 days' time elapsed between introduction of spleen cells and injection with virus.

Fig. 3 shows the relative growth patterns of polyoma virus in kidneys, livers and the salivary glands of thymectomized and litter mate control C57BL mice from 4 hours to 18 days after introduction of virus. It is clear that there are no striking differences in these growth curves nor are there any differences in the mean HI antibody titers measured from 4 to 30 days after introduction of polyoma virus (Table III).

Discussion. Surgical removal of the thymus at 3 days of age in C57BL, C3Hf/Lw, (Bl × C3H)F_1 and C3Hf/Bi mice has led to a strikingly increased frequency of parotid gland neoplasms especially if polyoma virus was administered within the first week of life. Also, the period of susceptibility to the oncogenic property of virus was extended to at least 30 days of age in C57BL mice. The

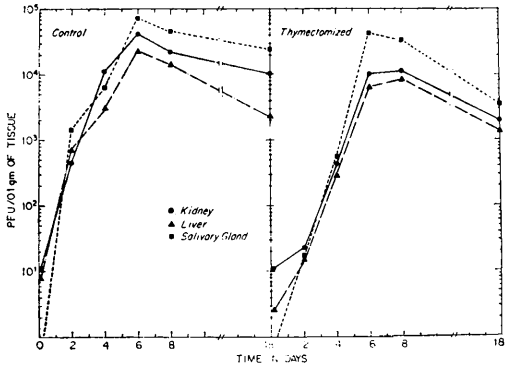

FIG. 3. Growth curves for LID-1 polyoma virus in 3-day thymectomized C57BL mice and their intact litter mates. LID-1 strain of virus (1×10^5 PFU) given at 7 days of age. Three mice in each group were sacrificed at various times from 4 hours to 18 days after infection for determination of virus concentration in various organs.

usual stigmata associated with neonatal thymectomy were not encountered in these 3-day thymectomized mice; nevertheless the methods used to restore the immunologically deficient neonate, introduction of adult syngeneic spleen cells (C57BL), neonatal thymic grafts or thymic tissue in cell-impermeable Millipore diffusion chambers were found effective in this study in restoring the potentiality to resist tumor induction by polyoma virus. The more effective restoration achieved in this study over that reported earlier using 1-day thymectomized mice(14) is in all probability related to the longer time interval between introduction of spleen cells and infection with virus.

The most plausible explanation for these results is that resistance to polyoma virus oncogenesis is related directly to a cellular immunity of the homograft type invoked against virus-specific new antigen(7,8) contained in the neoplastic cells. In the immunologically disturbed animal these "new" antigens are not capable of provoking an effective immunological attack on the cells transformed to neoplasia by polyoma virus. These clones of cells therefore grew progressively into frank neoplasms. In the immunologically competent animal, either intact or restored, neoplastic conversion occurs but such cells are aborted by a successful homograft-type reaction. No direct histologic evidence is at hand however to demonstrate such abortive conversions. Immunologic repair is accomplished in the thymectomized C57BL mouse by all of the 3 methods attempted here to the extent of permitting adequate homograft rejection and thus repressing the origin of neoplastic cells.

TABLE III. Average Hemagglutination-Inhibition (HI) Antibody Titers After Polyoma Virus Infection of C57BL Mice.*

Group	Days after infection with virus				
	4	10	15	21	30
Control	<100	250	465	1200	1600
Thymectomized	<100	100	500	1320	2000

* Six thymectomized mice and 6 matched litter mates infected with LID-1 polyoma virus (1×10^5 PFU/mouse) on day 7.

Conceivably, the absence of thymic tissue could enhance polyoma virus growth leading to an earlier and greater concentration of virus in the target organs. The highly oncogenic S-polyoma virus, for example, showed a 1000-fold greater titer at 5 days after early infection than did the M-polyoma low-oncogenic variant(29). It is clear from the results reported here that virus growth curves for LID-1 polyoma virus are similar in intact and thymectomized mice and that the virus growth pattern is not a significant factor in the increased sensitivity of thymectomized mice.

Previously(9,13) it was noted that antiviral antibody titers (HI) determined at 3 weeks or later following infection were not strikingly different in thymectomized and intact litter mates, confirming the work of others(11,12). In the present study HI antibody titers were measured several times from 4 days to 30 days after infection and, as suspected from the virus growth patterns, no significant differences were detected.

It is suggested by recent reports(29,30) that interferon plays some part in polyoma virus oncogenesis. Determinations of interferon levels were not made in the present study. However, according to the findings of Friedman and Rabson(29) the growth curves of virus in intact and thymectomized mice should differ if different levels of interferon are produced in the 2 groups. The fact that virus growth curves were similar in the intact and thymectomized mice in all 3 organs examined suggests that difference in interferon

production is not a major factor in increased sensitivity to polyoma virus.

Early thymectomy in the rat is associated with an increased sensitivity to polyoma virus. In this species a 4-fold increase in frequency, particularly of kidney sarcomas, has been reported following total thymic removal at birth and polyoma virus inoculation immediately after thymectomy(11). In the Syrian hamster two aspects of polyoma virus tumorigenesis appear to be affected: 1) liver hemangioma frequency is increased in those individuals thymectomized at birth, virus being given at 6 months of age and, 2) thymectomized hamsters appear to have more multiple tumors at sites involving: heart, kidney, and the subcutaneous connective tissues at the site of inoculation of virus(16). In neither the thymectomized rat nor the Syrian hamster, however, is there definitive evidence that increased sensitivity (decreased resistance) to polyoma virus oncogenesis has an immunologic basis, although the findings of Defendi and Koprowski(31) are highly suggestive.

The results presented here are consistent with the concept that in animals that are in a condition of immunologic inadequacy, as a result of early thymectomy, malignant conversion by polyoma virus is more readily achieved than in immunologically adequate animals. The immunologic deficit may indeed be quite subtle and not easily recognized grossly. The mice studied here that were thymectomized at 3 days showed little lymphoid depletion, they were capable of rejecting in normal fashion allogeneic skin grafts and of forming hemolysins to sheep erythrocytes and antiviral antibodies.

Summary. Thymectomy at 3 days of age in several inbred strains of mice and in an F_1 hybrid resulted in a strikingly increased frequency of neoplasms following infection with polyoma virus. Age susceptibility was extended to at least 30 days of age in highly resistant C57BL mice. The usual stigmata associated with thymectomy at birth were not found in the 3-day thymectomized mice. Nonetheless the methods used to restore immunologically deficient thymectomized neonates were also effective in restoring the capacity to resist polyoma virus tumor induction: adult syngeneic spleen cells, thymus tissue in Millipore diffusion chambers and syngeneic thymus grafts. Growth curves of polyoma virus in kidney, salivary glands and liver were quite similar in thymectomized and intact litter mates. Hemagglutination-inhibition antibodies determined periodically up to 30 days after infection were also similar in both groups. These results are discussed in terms of a concept involving virus-specific "tumor" antigens and the immunologic status of the animal.

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Acute Metabolic Effects of Nicotinic Acid in Alloxan Diabetic Dogs.* (30312)

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It has been demonstrated that nicotinic acid in high doses for a prolonged time has a cholesterol lowering effect in man(1). The mechanism of this action has not yet been fully elucidated(2). It was further demonstrated that nicotinic acid causes a rapid decrease in the plasma Free Fatty Acids (FFA) concentration and that it inhibits the increase of FFA produced by nor-epinephrine in fasting man(3). Recently it has been shown *in vitro*(4) that nicotinic acid inhibits FFA release from epididymal pads of alloxan diabetic rats and from adipose tissue taken from diabetic human subjects(5). Older reports have suggested that nicotinic acid has a hypoglycemic effect in diabetic patients(6).

The purpose of this paper was to determine if a depression of plasma FFA concentration was obtainable in alloxan-diabetic dogs and what relationship, if any, could be detected between blood sugar and concentration of FFA.

Materials and methods. Six previously alloxanized (90 mg/kg i.v.) diabetic mongrel dogs of both sexes, weighing 9-15 kg were used. They had a daily NPH insulin‡ requirement varying between 15 and 35 U. They were kept in metabolism cages on a regular diet and their daily urinary output, glycosuria and ketonuria were checked. Water was allowed *ad libitum*.

In preparation for each experiment, insulin was withdrawn for 3 days and food withheld for 16 hours. The day of the experiment, all animals showed ketonuria (from traces to mild), in addition to glycosuria (30 to 140 g/24 hr). Two weeks were allowed as an interval between 2 experiments performed in the same dog. The animals were not anesthetized during the tests. Nicotinic acid (50 mg/dog) or isotonic saline was administered in a peripheral vein. Blood samples obtained from another peripheral vein were stored in heparinized tubes kept in an ice bath. Aliquots for blood sugar determinations(7) were removed and the rest of the blood was centrifuged at 4°C. Plasma FFA determinations (8) were carried out immediately after centrifugation. Plasma total cholesterol was measured according to Abell(9).

Results. Results are presented in Table I and Fig. 1. Table I gives the average pre-

TABLE I. Mean Pre-Injection Values in Diabetic Dogs (± S.E.). (12 experiments)

Blood glucose	284 ± 16 mg %
Plasma total cholesterol	232 ± 13 mg %
Plasma FFA	1340 ± 130 μEq/l

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