lowed by reattachment under unstrained conditions. Except at extreme lengths, where there is a large contribution from connective tissue, this could account for most of the viscoelasticity in resting smooth muscle. It follows that there appear to be three effects associated with different intracellular calcium concentrations: (i) at very low levels the calcium-dependent resistance to stretch disappears; (ii) at higher levels crossbridge attachment occurs, manifested as viscoelasticity; and (iii) at still higher levels there may be activation of actomyosin adenosine triphosphatase and crossbridge cycling manifested as tone or, following normal excitation-contraction coupling, tension development and shortening.

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Activation of Transplant Immunity: Effect of Donor Leukocytes on Thyroid Allograft Rejection

Abstract. *The survival of thyroid allografts in mice was prolonged by either holding the grafts in in vitro culture for 20 to 27 days or by cobalt-60 irradiation of the donor 2 days before transplantation with or without the intravenous injection of colloidal carbon just before removing the thyroid from the donor. In both cases the rejection process was restored by an intravenous injection of recipients with living peritoneal exudate cells (50 to 80 percent macrophages) syngeneic to the thyroid donor.*

The survival of both thyroid and ovarian transplants in allogeneic mice has been prolonged by first culturing the grafts for several weeks in vitro (*1, 2*). The finding by Summerlin and co-workers of a similar prolongation of cultured skin grafts (*3*) has not been confirmed partly because of failure of cultured skin grafts to revascularize even when syngeneic to the recipient (*1*). Two alternative mechanisms have been proposed for the prolonged survival of thyroid and ovarian grafts. (i) A modification of tissue antigens occurs during the culture in vitro resulting in a loss of tissue antigenicity. (ii) Some of the leukocytes trapped in the graft are essential for the activation of recipient T lymphocytes (thymus derived), and are gradually lost during the culture in vitro.

In relation to this second proposition, we have proposed that the activation of specific T lymphocytes is the function of a living cell with special properties (*4*) and have reported evidence that in mice the macrophage is the most efficient if not the only cell with this stimulating activity (*5*). Others have reported similar evidence for guinea pig and human macrophages (*6*). The importance of passenger leukocytes in transplant immunity has already been demonstrated (*7*).

We reasoned that, if the loss of tissue leukocytes were responsible for the prolonged survival of cultured organ grafts, then the injection into the recipient of leukocytes syngeneic to the graft should activate specific transplant immunity and cause graft rejection. In contrast, if the loss of tissue antigens was responsible for the prolonged survival, the injection of leukocytes would have little effect. We also used an alternative method of reducing the number of blood leukocytes in the graft: gamma radiation of the donor 2 days before removal of the organs. This treatment also prolonged allograft survival. With both the cultured and the irradiated grafts we found that the intravenous injection of living peritoneal exudate cells (rich in macrophages and lymphocytes), if syngeneic to the graft, stimulated its rejection.

Individual lobes of BALB/c (H-2d) mouse thyroid glands were placed on rafts in 60-mm organ culture dishes (Falcon) containing Eagle's minimum essential medium (MEM) supplemented with 10 percent fetal calf serum, penicillin, and streptomycin. The cultures were maintained for 20 to 27 days at 37°C in a humid atmosphere of 95 percent O_2 and 5 percent CO_2. The organ culture medium was changed three times each week.

Thyroids were transplanted by placing individual lobes under the left kidney capsule. Thyroidectomy was performed on recipients of cultured thyroids and their controls (Table 1), but not on the other recipients of a direct transplant (Tables 2 and 3). After several weeks in culture the ability of transplanted thyroid glands to take up iodine was considerably reduced compared to direct transplants, and thyroidectomy of the recipient was required to stimulate transplant function to detectable levels. The function of the transplanted thyroids was determined by an intraperitoneal injection of recipient mice with 0.25 μc of carrier-free ^{125}I. The recipient was killed 24 hours later, and both kidneys were removed and placed in counting tubes containing formalin in saline. The radioactivity in the left kidney, which contained the thyroid transplant, as well as that in the control right kidney, was then counted in a gamma counter. Some groups of recipients were placed on low iodine food for 3 days prior to the injection of ^{125}I. This diet increased the uptake of radioactivity in the grafted and control kidneys approximately fivefold, but the ratio of radioactivities in the grafted to the control kidneys was not significantly altered. All transplants of cultured thyroid were examined histologically after counting. Selected kidneys receiving direct transplants were similarly examined, including all grafted kidneys whose radioactivity was not significantly above its control.

Peritoneal exudate cells (PEC) were obtained by washing the peritoneal cavities of normal mice with approximately 10 ml of MEM each. The cells were washed twice with MEM and counted. Electron micrographs showed that 50 to 80 percent of the cells so obtained had the typical lysosomes and ruffled membranes of macrophages. The other cells were mostly small lymphocytes.

All 11 uncultured BALB/c (H-2d) thyroids were completely rejected 15 days af-

ter transplantation into CBA (H-2k) recipients (the ratio of grafted to control kidneys was 0.91 ± 0.03; mean ± standard error) while 10 of 12 cultured allografts showed significant function for this period (mean ratios, 4.9 and 10.3) (Table 1). The intravenous injection of CBA recipients with 10^5 CBA PEC at the time of transplantation had no effect on the survival of BALB/c thyroid (mean ratios, 7.5 and 33). However the injection of 10^5 BALB/c PEC into such recipients resulted in the rejection of all transplants (mean ratios, 0.88 and 1.03). Histologic examination showed little or no cellular infiltration and normal structure in the surviving groups while complete destruction of normal structure was observed in the rejected groups. These findings show that organ culture prior to thyroid transplantation reduces allograft immunogenicity, but that the cultured tissue retains its antigens and can be rejected when the recipient's immune system is activated by leukocytes syngeneic to the tissue transplant.

Uncultured thyroids were transplanted directly into untreated mice (Table 2). In the first group of experiments, A/J (H-2a) thyroids were placed in BALB/c (H-2d) mice. Only one of five grafts survived 10 days when no treatment was given to the donor (group A-1), and the mean ratio of counts in grafted to control kidneys was 6.7 ± 5.6. When 0.3 ml of a 1 : 5 dilution of a stock colloidal carbon suspension (Pelikan C 11/143a, 10 percent carbon) was injected intravenously into donor mice immediately before they were killed, grafts survived well for 10 days (group A-2), and the mean ratio was 64 ± 9; but only one of five grafts survived 20 days (group A-3). The additional treatment of donor mice with 750 rads of ^{60}Co radiation 2 days before the animals were killed produced a further increase in graft survival (groups A-4 and A-5).

The second group of experiments reported in Table 2 shows the survival of CAF_1 (BALB/c × A/J) thyroids in BALB/c mice. The effect of carbon and gamma radiation given to donors is less striking here than with A/J grafts because of the high 13-day survival of CAF_1 grafts from untreated donors (group B-1). However, the combination of carbon and gamma radiation resulted in consistent graft survival at 30 and 40 days (group B-5), whereas grafts from untreated donors survived 20 days much less regularly (group B-2).

The third group of experiments was performed with thyroid grafts from BALB/c mice treated with ^{60}Co radiation and carbon. When these grafts were placed in the highly incompatible C_{57}BL (H-2b) mice, nine out of ten showed insignificant function by 13 days and the remaining one had a low ratio of counts in grafted to control kidneys (6.9). This shows that, though the combination of gamma rays and carbon was effective in prolonging the survival of the relatively weak rejection of A/J grafts by BALB/c mice, the donor treatment was ineffective against the stronger allograft rejection of BALB/c grafts by C_{57}BL mice. Thus, the ^{60}Co and carbon treatment is less effective in reducing the immunogenicity of grafts than the more tedious in vitro culture method, since Lafferty *et al.* found good survival (six out of eight) of cultured BALB/c grafts in C_{57}BL mice (*1*). This may be due to the fact that macrophages are resistant to gamma radiation and only a few such cells are required to activate transplantation immunity to the most highly incompatible grafts.

On the basis of the above results more experiments were performed to determine the importance of living leukocytes of the donor strain to the induction of transplant immunity. Thyroids from donor CAF_1 mice were chosen because they survived regularly when donors were treated with

Table 1. Effect of injection of peritoneal exudate cells (PEC) into thyroidectomized CBA mice with BALB/c thyroid allografts.

Group	Culture (days)	Cells injected into recipient	^{125}I uptake* (dpm)	Ratio†	Function‡
1	None	None	−74 ± 29	0.91 ± 0.03	0/11
2	21§	None	14,000 ± 3,500	4.9 ± 0.6	4/6
3	26	None	5,600 ± 600	10.3 ± 0.8	6/6
4	20§	10^5 CBA PEC‖	15,500 ± 3,400	7.5 ± 1.6	6/7
5	26	10^5 CBA PEC	15,900 ± 3,500	33 ± 8	7/7
6	20§	10^5 BALB PEC	−66 ± 20	0.88 ± 0.03	0/6
7	27	10^5 BALB PEC	4 ± 72	1.03 ± 0.05	0/5

*Mean disintegrations per minute (dpm) in grafted kidney on day 16 less those in control kidney ± standard error. The efficiency of gamma counter was 47 percent. †Ratio of disintegrations per minute in grafted to control kidneys. ‡Ratio of the number of mice with at least four times as many counts in the grafted kidney as in the control to the total recipient mice in group. Two mice in group 2 and one mouse in group 4 had ratios between 2.8 and 4. The highest ratio in groups 1, 6, and 7 was 1.2. §Recipients in groups 2, 4, and 6 were placed on low iodine diet 3 days before injection of ^{125}I. ‖Peritoneal exudate cells.

Table 2. The survival of thyroid allografts and semiallografts in untreated recipients. Abbreviations: X, ^{60}Co irradiation; C, carbon; and dpm, disintegrations per minute.

Group	Donor	Treatment of donor	Recipient	Day of ^{125}I	Mean net dpm	Mean ratio	Graft survival*
A-1	A/J	None	BALB/c	10	1,685 ± 1,661	6.7 ± 5.6	1/5
A-2	A/J	C†	BALB/c	10	4,077 ± 483	64 ± 9	4/4
A-3	A/J	C	BALB/c	20	440 ± 427	10 ± 8	1/5
A-4	A/J	X + C‡	BALB/c	10	4,776 ± 1,369	92 ± 26	5/5
A-5	A/J	X + C	BALB/c	20	2,660 ± 1,499	40 ± 19	4/5
B-1	CAF_1	None	BALB/c	13	3,305 ± 945	57 ± 16	5/6
B-2§	CAF_1	None	BALB/c	20	7,385 ± 4,917	34 ± 23	3/8
B-3	CAF_1	C	BALB/c	13	3,129 ± 1,655	62 ± 33	3/4
B-4	CAF_1	X‖	BALB/c	13	7,158 ± 1,234	158 ± 60	5/5
B-5§	CAF_1	X + C	BALB/c	30;40	18,349 ± 3,794	177 ± 78	7/7
C-1§	BALB/c	X + C	C_{57}BL	13	437 ± 308	1.9 ± 0.6	1/10
C-2	BALB/c	X + C	BALB/c	10	3,748 ± 856	52 ± 13	5/5

*Ratio of numbers of mice with at least four times as many counts in grafted kidney as in the control kidney to the total number of mice in the group. †Colloidal carbon injection immediately before the animals were killed. ‡750 rads of ^{60}Co irradiation and colloidal carbon injection. §Low iodine food given to recipients for 3 days before injection of ^{125}I. ‖750 rads of ^{60}Co irradiation only.

Table 3. The 14-day survival in BALB/c mice of thyroid semiallografts from CAF_1 donors pretreated with gamma ray and carbon.

Group	Cells injected	Mean ratio*	Number with ratio	
			> 4	> 16
A	None	96 ± 12	36/36	34/36
B	10^3 CAF_1 PEC	2.7 ± 0.7	5/29	0/29
C	10^3 BALB PEC	144 ± 9	5/5	5/5
D	10^3 C_{57}BL PEC	47 ± 15	5/5	4/5
E	Killed CAF_1 PEC†	81 ± 12	16/16	16/16
F	CAF_1 PEC in CFA‡	33 ± 13	6/9	4/9

*Ratio of counts in grafted kidney to control kidney. The net disintegrations per minute have been omitted because of the extreme variability of this figure with diet. However, all mice were treated with low iodine food for 3 days before injection of ^{125}I. †See text for different methods of killing cells. ‡10^6 CAF_1 PEC sonicated in complete Freund's adjuvant and injected into recipients 2 weeks before transplantation.

750 rads of ^{60}Co 2 days before grafting and injected with carbon immediately before they were killed. The CAF_1 grafts in BALB/c mice had the additional advantage that the stimulation of T cells in this combination is in one direction only since the CAF_1 T cells in the graft would not be expected to be stimulated by host cells from the parental BALB/c strain.

On day 13, ^{125}I was injected and the radioactivity in the kidneys was counted on day 14. Of 36 CAF_1 grafts placed in BALB/c mice without injection of PEC (group A), all showed function at 14 days with a mean ratio of radioactivities in grafted and control kidneys of 96 ± 12 (Table 3).

Twenty-nine similar grafts placed in BALB/c recipients injected with 1000 CAF_1 PEC (group B) showed a significant function in only five grafts with a mean ratio of 2.7 ± 0.1. The highest ratio in the recipients of CAF_1 PEC was 16. Only two of 36 uninjected recipients had a lower ratio; of these ratios one was 8 and the other 14. The injection of only 100 CAF_1 PEC into BALB/c mice bearing CAF_1 grafts produced only occasional rejection (data not shown). Five CAF_1 grafts in BALB/c recipients injected with 1000 BALB/c PEC (group C) showed an average ratio of 144 ± 9, which was not significantly different from the uninjected group from the same experiment. The injection of 1000 $C_{57}BL$ PEC into similar recipients (group D) resulted in a mean ratio of 47 ± 5, which was a significant reduction in graft function compared to uninjected or BALB/c injected controls, but much more function than seen in the group injected with donor CAF_1 PEC.

The injection of killed donor type PEC into 16 BALB/c recipients at the time of transplanting CAF_1 thyroid grafts (group E) did not have a significant effect. The mean ratio in this group was 81 ± 12. The cells injected were as follows: 10^4 cells heated at 56°C for 1 hour (three recipients), 10^4 cells treated with 1 percent formalin for 1 hour (three recipients), 10^4 cells sonicated for three 10-second intervals in a Branson Sonifier (three recipients), 10^6 sonicated cells (four recipients), and 10^6 cells sonicated in complete Freund's adjuvant (three recipients). The injection of the cells in Freund's adjuvant 2 weeks before grafting (group F) had mixed effects. On histologic examination three of the grafts showed no function and almost complete destruction. Four of the grafts showed excellent function (mean ratio of 64) and little or no infiltration of mononuclear cells. The other two showed moderate function and infiltration.

Our data extend previous studies of the effect of organ culture on thyroid allograft survival (*1, 2*). Lengthening the organ culture period to more than 3 weeks results in a situation where cultured allografts are indistinguishable from isografts 16 days after transplantation. The failure of the cultured tissue to stimulate its own rejection is not due to an inaccessibility to circulating lymphoid cells or an incapacity of the latter to recognize the cultured tissue as foreign. Once the recipient's immune system has been activated by living peritoneal cells syngeneic to the cultured allograft, this tissue is promptly rejected.

These findings are consistent with the concept that a living stimulator cell is required for T cell activation (*3*). It would appear that during organ culture this stimulator cell either dies or is inactivated. Irradiation of the thyroid donor 2 days prior to the removal of the thyroid for transplantation with or without carbon injection of donor also prolonged the survival of this tissue in allogeneic and semiallogeneic recipients. Since gamma radiation reduces blood leukocyte counts, this finding provides further support for the concept that passenger leukocytes carried in the transplant play an important role in the activation of the allograft response. Alternatively, it is possible that the effect of both culture and gamma radiation is to change the antigen on tissue cells from a complete antigen to that of a hapten. If so it is necessary to also postulate that heating, formalin treatment, and sonication of injected cells all have the same end result: reduction of antigenicity.

Although our data are consistent with our working hypothesis that macrophages have a unique role as stimulator cells, the data provide no evidence that other cells, such as lymphocytes, are not equally effective. Although the technique of irradiating the donor was less effective than culturing in strain combinations with strong rejection reactions, it was much easier and quicker than culturing the thyroid because thyroidectomy of recipients was not required and the long time of culturing grafts was avoided. This modified technique should be useful in elucidating which cell or cells are important in the induction of allograft immunity.

There are many advantages of thyroid grafting for the study of transplant immunity. (i) The small size ensures a minimum number of trapped leukocytes to start with and a relatively easy removal of those remaining. (ii) The function of the graft can be objectively and quantitatively assayed by its ability to take up radioactive iodine. (iii) The cells of the thyroid are not dividing and are thus relatively resistant to gamma rays. (iv) The function of the thyroid cells is maintained for long periods of culture in vitro. (v) The thyroid has a unique and characteristic appearance, and the graft can be positively identified for several days after the function has been completely destroyed.

The requirement of a stimulator cell for T cell activation provides an explanation for the specificity of T cell phenomena where the activity of primed T cells is assayed (*8*). If the T cell must interact with antigen on the surface of a stimulator cell before it can be induced, it is possible that the T cell receptor "sees" both the antigen and part of the surrounding cell surface components. Thus, these primed T cells will now interact most efficiently with complexes of the type to which they were initially primed. Cells primed in a syngeneic system will be specific for antigen in combination with a syngeneic or semiallogeneic cell. No such restriction will be observed when allogeneic stimulator cells provide the inductive stimulus, as is the case when cytotoxic cells are generated in mixed leukocyte culture. It may be for this reason that living allogeneic stimulator cells are much more efficient in the induction of allograft immunity than antigen presented on dead cells, either alone or emulsified in complete Freund's adjuvant. Antigen presented on allogeneic stimulator cells would activate T cells specific for the allogeneic cells. Antigen derived from cells unable to stimulate themselves would have to be presented to host T cells via the host's own stimulator cells, and thus the T cell response induced by such antigen may be specific for this material in combination with a cell of host genotype. Such T cells may be relatively inefficient in their interaction with the same antigen presented on the surface of allogeneic target cells. This effect would be similar to the allogeneic restriction observed in the T cell mediated killing of virus infected cells (*9*).

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Role of the Generative Cell in Androgenesis in Henbane

Abstract. *When anthers of henbane containing uninucleate pollen grains were cultured, a large number of embryoids originated exclusively from the division of the generative cell. In a small proportion of pollen grains, both generative and vegetative cells contributed to embryoid formation. Embryogenesis by segmentation of the vegetative cell alone was rarely observed.*

When immature anthers of certain angiosperms are cultured at an appropriate stage of development in a defined nutrient medium, the pollen (*1*) may form sporophytic plants with the haploid number of chromosomes. Cell divisions are initiated in a certain proportion of the pollen grains contained in excised and cultured anthers, leading to the formation of embryoids. The latter develop through stages reminiscent of those of true zygotic embryos before they emerge as plantlets outside the anther wall. This phenomenon, known as androgenesis, has been reported to occur readily in plants belonging to Solanaceae (*2*).

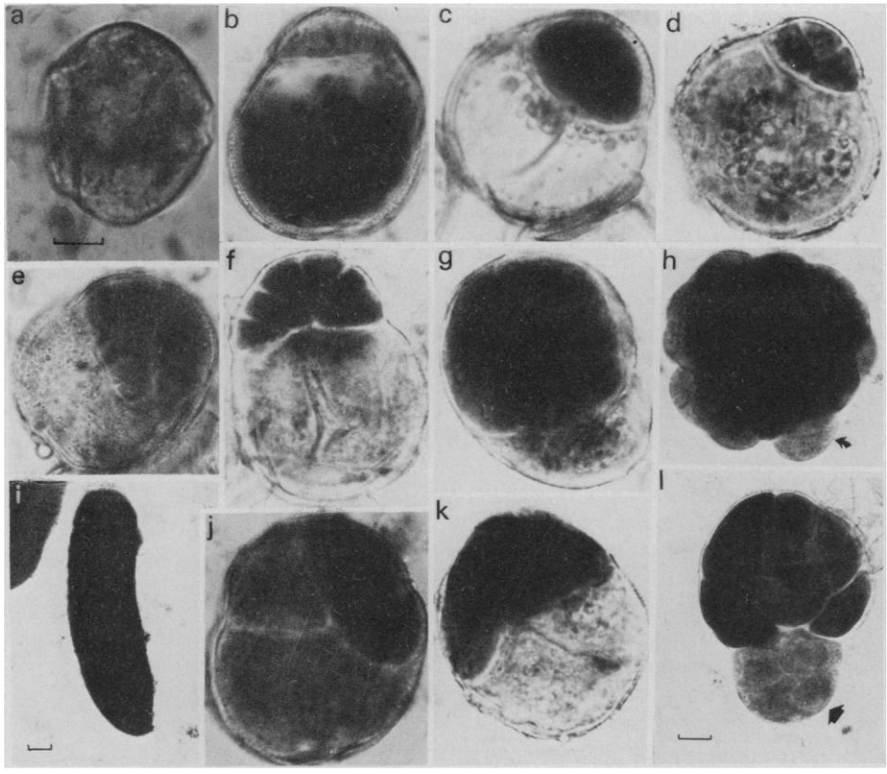

Fig. 1. (a) Uninucleate pollen grain of henbane 24 hours after culture; the nucleus is in division. (b) Large densely stained vegetative cell and a small generative cell with granular nucleus, following the first pollen mitosis. The nucleus of the vegetative cell is not seen; 72 hours after culture. (c) Contrasting staining patterns of the nonembryogenic vegetative cell and the embryogenic generative cell; 72 hours after culture. (d) Division of the generative cell in the embryogenic pathway, forming a quadrant; 96 hours after culture, (e and f) Planes of division of the generative cell to form embryoids. The large vegetative cell has not divided; 144 hours after culture. (g) A globular embryoid originating from the generative cell, still enclosed within the exine. The basal cell is the partially crushed vegetative cell; 168 hours after culture. (h) A later stage of globular embryoid, after release from the exine. Arrow points to the vegetative cell, which persists as a suspensor-like organ; 168 hours after culture. (i) A torpedo-shaped embryoid. No trace of the suspensor is seen; 216 hours after culture. (j) Division of the vegetative and generative cells; 72 hours after culture. (k) An embryoid originating from the generative cell. The vegetative cell has divided once; 120 hours after culture. (l) A globular embryoid originating from the generative cell. The vegetative cell has divided to form a multicellular suspensor-like organ (arrow); 192 hours after culture. The scale (0.1 mm) in (a) refers to (a) to (e), (j), and (k); the scale (0.1 mm) in (l) refers to (f), (g), (h), and (l); the scale in (i) is 0.1 mm.

Several workers (*3*) have established that in cultured anthers, following the characteristic asymmetric pollen mitosis, embryoid formation results from the activity of the large vegetative cell, while the small generative cell gradually degenerates. Not uncommonly, the pollen grain might form two equal cells after the first mitosis, and both cells contribute to the formation of the embryoid (*4–6*). Still a third pathway, which involves fusion between the vegetative and generative cells and their subsequent division in a complex manner, has also been described (*6*). These results indicate that the generative cell has practically no role or only a limited role in androgenesis. This is surprising, since in the normal ontogeny of the male gametophyte the generative cell gives rise to gametes while the nucleus of the vegetative cell degenerates or survives as a vestigial structure in the pollen tube (*7*). Observations presented in this report show that in anther cultures of henbane (*Hyoscyamus niger*, annual variety), continued division of the generative cell alone following the first pollen mitosis may account for a significant proportion of the embryoids formed. Details of culture conditions for obtaining optimum yield of embryoids from anthers of henbane have been described elsewhere (*8*).

Anthers cultured at the uninucleate pollen stage were collected at intervals of 24 hours for 10 days and examined by squashing in acetocarmine. Pollen grains at the late uninucleate stage or in the process of division at the time of culture were generally binucleate by 24 to 48 hours after culture. At this time it was possible to separate a group of densely staining, slightly enlarged, potentially embryogenic pollen from the rest of the population. Observations of anthers on subsequent days showed that many potentially embryogenic pollen grains had become abnormally enlarged and full of starch. The starch-filled grains as well as the majority of the binucleate pollen that stained weakly with acetocarmine were nonembryogenic.

Division of the pollen grain in the embryogenic pathway was observed as early as 48 to 72 hours after culture. After the first pollen mitosis, wall formation occurred, delimiting a small generative cell with granular nucleus and a large vegetative cell with diffuse nucleus (Fig. 1, a and b). The cytoplasm of the large cell also stained intensely with acetocarmine, as it does in a typical vegetative cell (*9*). As Fig. 1c illustrates, the staining reaction of the vegetative cell was transient and it dis-