SURVIVAL OF SKIN HOMOGRAFTS IN DOGS INJECTED WITH ^{109}Pd-PROTOPORPHYRIN

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Intravenous administration of ^{109}Pd-protoporphyrin is demonstrated to localize in peripheral lymphoid tissue, thus accomplishing selective radiation destruction of lymphatic tissues responsible for homograft rejection. In two dogs, so treated, skin homografts continue healthy for 5 months after transplantation as shown by lack of acute rejection, physical characteristics, and chromosome markers.

Homograft rejection is a function of competent lymphatic tissue (*1*). The present work was undertaken to develop methods for selective lymphatic ablation for suppression of the homograft rejection response.

An invariably fatal "wasting" disease is associated with overall diminution in lymphatic tissue (*2,3*). Therefore, any technique developed for abrogation of the homograft rejection response should selectively destroy those lymphatic cells committed to the rejection of the homograft but spare immunologically uncommitted lymphatic tissue. Lymphocytes within the thymus and the bone marrow appear to be relatively uncommitted in terms of their ability to react to foreign antigens (*4–9*). Many of the cells contained in lymph nodes and spleen appear to be committed and are able to react to foreign antigens (*10*). For these reasons, we felt it desirable to have an agent which could deliver high doses of radiation to lymph nodes and spleen while still sparing the thymus and bone marrow. Furthermore, any radioisotope chosen for selective lymphatic irradiation should deliver its radiation locally, i.e., an alpha, beta, or low-energy gamma-emitting radionuclide.

We have previously demonstrated that ^{109}Pd (a predominantly beta-emitting radionuclide) when chelated to protoporphyrin IX and administered i.v. to dogs, has a much greater affinity with peripheral lymphoid tissue than with thymus and bone marrow (*11*). An investigation therefore was initiated to determine the efficacy of ^{109}Pd-protoporphyrin in suppressing homograft rejection. We report here the results of this investigation and show the usefulness of ^{109}Pd-protoporphyrin in the suppression of skin homograft rejection in dogs.

MATERIALS AND METHODS

Protoporphyrin IX was obtained from Calbiochem (Los Angeles, Calif.), $^{109}PdCl_2$ (6 Ci/gm) was obtained from the Nuclear Science Corp. (Pittsburgh, Pa.), and the ^{109}Pd-protoporphyrin complex was prepared according to the method of Theorell (*12*). Nickel-63 acetate (5 Ci/gm) was obtained from the New England Nuclear Corp. (Boston, Mass.), and the ^{63}Ni-protoporphyrin complex was prepared according to the method of Taylor (*13*). In all experiments the sodium hydroxide-soluble fraction of the radioactive metalloporphyrin complex was mixed with uncomplexed protoporphyrin IX making 120 mg the total amount of porphyrin (protoporphyrin IX plus its metallocomplex). This porphyrin mixture was administered i.v. to beagles weighing 19–22 lb.

In one experiment, four dogs were injected i.v. with 50 μCi of either ^{109}Pd or ^{63}Ni chelated to protoporphyrin IX. Plasma samples were obtained for determination of radioisotope concentration. One day after injection, the dogs were killed and the concentration of radioactivity in their tissues determined.

In a second experiment, one dog received 500 μCi of ^{63}Ni-protoporphyrin IX i.v. One day later the dog was killed and its tissues removed and prepared for determination of radioisotope localization by autoradiography. Autoradiography was performed according to the method of Kopriwa (*14*).

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In a third experiment, one dog received 5 mCi/kg of ^{109}Pd-protoporphyrin IX i.v. Three days later the dog was killed and its tissues removed for histologic examination.

In a fourth experiment, two dogs were injected with 14 mCi/kg of ^{109}Pd-protoporphyrin each. Three days later the dogs received full-thickness skin homografts from unrelated donor dogs. As a control experiment, full-thickness skin was taken from these ^{109}Pd-protoporphyrin dogs and transplanted onto their respective donor dogs. As an additional control, autotransplants of skin were performed on all dogs. Dogs injected with ^{109}Pd-protoporphyrin were chosen such that the skin over the dorsum of their necks, the site chosen for transplantation, was nonpigmented and white-haired. On the control animals, the same region was pigmented and black-haired. Thus, ^{109}Pd-protoporphyrin-injected dogs received pigmented black-haired skin homografts placed within a nonpigmented white-haired area of the skin. In one of these experiments involving reciprocal skin transplants, both the experimental and control animals were female. In the other experiment, the ^{109}Pd-protoporphyrin-injected dog was a female and the control dog was a male. In the latter case two skin homografts were performed on each dog; one of the skin homografts was removed 5 months later for determination of chromosomal sex.

The procedure used for skin homografting consisted of shaving the dorsal aspect of the neck, and after cleaning the area, surgical excisions of the skin were made with a dermal trephine 2 cm in diameter (*15*). The under surfaces of the skin pieces were trimmed of excess fat; then the grafts were rotated 180 deg (hairs pointed anteriorly) prior to suturing in place and application of dressing.

The method used for sex chromosome analysis consisted of surgically removing 1 cm × 1 cm full-thickness skin biopsies from the male donor, the female recipient, and from the homograft site. The samples were minced; pieces were placed under cover slips into culture dishes containing medium and then incubated at 37°C in 10% CO_2. Four weeks later cellular outgrowth was sufficient for harvesting. Velban added to each culture effected mitotic arrest; 5 hr later the cells were hypotonically swollen and fixed. The cover slips were removed and cells air-dried in situ were stained with Giemsa. The chromosome preparations were observed to determine the total number of chromosomes and the number of X chromosomes present in each cell.

In the transplantation experiments, complete blood counts, serum BUN, serum creatinine, serum protein, A/G ratio, and serum bilirubin were obtained prior and at various times subsequent to the administration of ^{109}Pd-protoporphyrin.

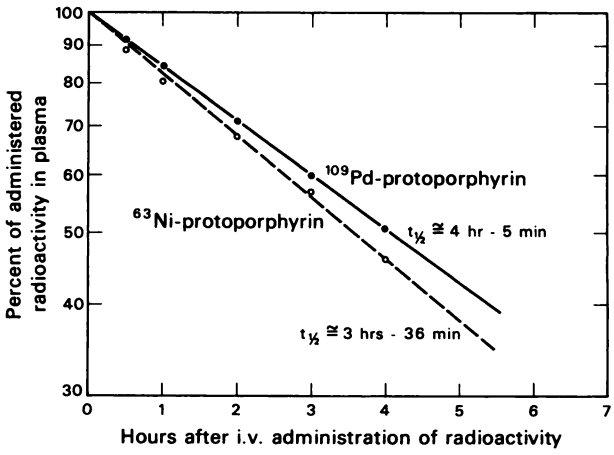

FIG. 1. Disappearance of radioactivity from plasma of dogs injected with either ^{63}Ni or ^{109}Pd-protoporphyrin complexes.

RESULTS

Figure 1 shows the pattern of disappearance of radioactivity from the plasma of dogs injected i.v. with either ^{109}Pd or ^{63}Ni-protoporphyrin complexes. Both radioactive metalloporphyrins gave similar plasma clearance patterns. Table 1 shows the tissue distribution of radioactivity in these dogs. A similar tissue-distribution pattern is observed following the administration of both radioactive metalloporphyrins. Since the 0.067-MeV beta particle emitted by ^{63}Ni is ideal for autoradiographic studies whereas the beta particle and gamma energies of the various Pd radioisotopes are not so well-suited for autoradiography, we used the ^{63}Ni-protoporphyrin to determine

TABLE 1. TISSUE-TO-MUSCLE RATIO 1 DAY FOLLOWING I.V. ADMINISTRATION OF RADIOACTIVE METALLOPORPHYRINS TO DOGS

Tissue	Average tissue/muscle ratio in 2 dogs injected with ^{63}Ni-protoporphyrin	Average tissue/muscle ratio in 2 dogs injected with ^{109}Pd-protoporphyrin
Liver	190	220
Mesenteric lymph node	172	220
Popliteal lymph node	116	131
Renal cortex	64	65
Spleen*	32	44
Bone marrow	36	46
Adrenal	26	21
Duodenal mucosa	14	12
Muscle	1	1

* At autopsy the spleen of these dogs was markedly enlarged, probably due to the vascular pooling after barbiturate administration.

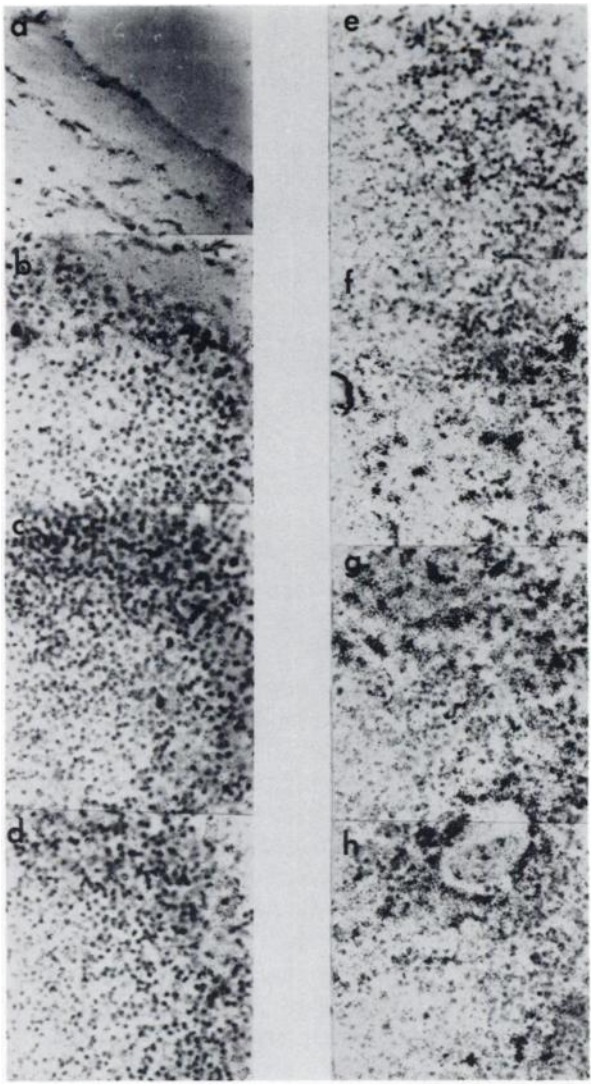

FIG. 2. Autoradiographs of section of lymph node removed from dog 1 day after i.v. injection of 500 μCi of ^{63}Ni-protoporphyrin. Figure sequentially shows regions of lymph node starting from capsule in panel (A) to hilar medulla in panel (H).

autoradiographically the site of localization of radioactive metalloporphyrins within the lymph node. In this way we determined the region(s) within the lymph node that were likely to be affected by irradiation from localized radioactive metalloporphyrins such as the ^{109}Pd-protoporphyrin. Figure 2 shows that the highest concentration of ^{63}Ni-protoporphyrin is found in the reticuloendothelial cells of the sinusoids. The latter are most abundant in the medulla, less so in the paracortical area, and least abundant in the cortex.

Considering the results of the autoradiographic studies and the 1-mm average range of the ^{109}Pd beta particle (*16*), one would predict that the lymphocytes in the medulla and the paracortical area (especially paracortex regions adjacent to the medulla) would be most severely affected following the administration of high-activity ^{109}Pd-protoporphyrin. Figure 3A shows the marked depletion of lymphocytes in the medulla and paracortical area of the lymph node from a dog injected with 5 mCi/kg of ^{109}Pd-protoporphyrin. Figure 3B shows a normal lymph node from a control dog. The spleen also showed marked lymphocyte depletion following the administration of 5 mCi/kg of ^{109}Pd-protoporphyrin; no alterations were observed in other tissues. This result increases the potential value of ^{109}Pd-protoporphyrin for suppression of homograft rejection since the periarteriolar lymphoid tissue of the spleen as well as the paracortical area of the lymph node have been implicated in the mediation of homograft rejection (*17,18*).

Our initial results with skin homografting are depicted in Figs. 4 and 5 and Table 2. Originally we selected four criteria for evaluating homograft survival: absence of acute rejection and continued healthy graft appearance, retention of donor skin pigmentation, retention of donor hair color, and reversal of direction of hair growth in homografted skin. From results obtained with autotransplantation, it became clear we could not depend on hair growth for our evaluations. In most instances hair growth was not observed in skin autotransplants; when present it was extremely sparse. This could be attributed to loss of hair follicles when trimming excess fat from the excised skin or to delay in hair regrowth which sometimes begins 6–12 months after homografting (*19*). Figure 4 shows black-pigmented donor skin grafted into the nonpigmented site of a dog injected with 14 mCi/kg of ^{109}Pd-protoporphyrin. The homografted skin has retained its black pigmentation 7 months after transplanta-

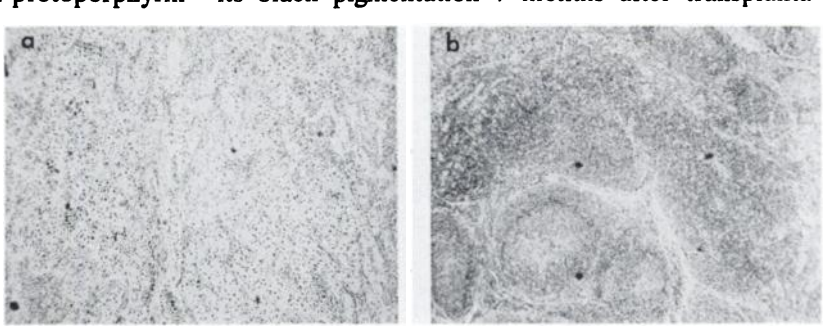

FIG. 3. (A) Histologic section of mesenteric lymph node removed 1 day after administration of high-activity ^{109}Pd-protoporphyrin and (B) similar section of mesenteric lymph node from normal control dog.

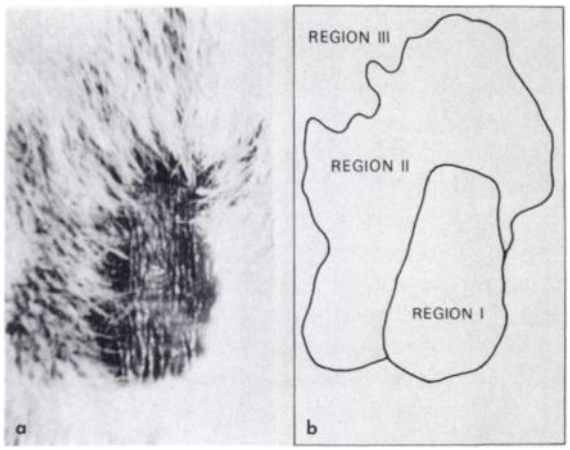

FIG. 4. Black pigmented donor skin (denoted Region I) grafted into nonpigmented site of dog injected with high-activity ^{109}Pd-protoporphyrin. In this dog pigment has spread (Region II) from homograft to surrounding originally nonpigmented skin of recipient dog. Region III is nonpigmented host skin.

tion. In this dog the pigment spread from the homograft to the surrounding nonpigmented skin. The phenomenon of pigment spread has been described previously (*20*). The reciprocal skin graft from this ^{109}Pd-protoporphyrin-injected dog to the donor dog underwent acute rejection 11 days after transplantation with subsequent contraction and scarring. These results are presumptive evidence for survival of skin homografts in dogs treated with ^{109}Pd-protoporphyrin. To confirm this, we added male chromosome status in the homograft as another criterion for homograft survival. As can be seen in Fig 5, 5 months after grafting pigmented black-haired skin from a male donor to the nonpigmented white-haired site of a female injected with 14 mCi/kg of ^{109}Pd-protoporphyrin, chromosomal analysis demonstrates the presence of male cells in the homograft site. Figure 6 shows a female skin cell of the recipient dog obtained from a site near to the homograft. Table 2 demonstrates that of the 82 cells with the diploid number of 78 chromosomes obtained from the homograft, 65.9% had one X and 30.5% had two X chromosomes; 92.5% of diploid cells from the male donor dog had a single X chromosome and none had two X chromosomes; and 97.4% of the female recipient's diploid cells had two X chromosomes and none had a single X chromosome. A reciprocal skin graft from the ^{109}Pd-protoporphyrin-injected dog to the control dog underwent rejection with subsequent marked contraction and scarring 23 days after transplantation. This did not allow obtaining an adequate number of cells from the control homograft for tissue culture and chromosome analysis.

Following the administration of 14 mCi/kg of ^{109}Pd-protoporphyrin to dogs, there was an initial drop in the absolute lymphocyte count to less than 6% of the pretreatment value. The absolute lymphocyte count returned to normal 2 weeks following the administration of radioactivity. Other hematologic parameters (red cell count, platelet count, and granulocyte count), kidney function tests (BUN, creatinine), and liver function tests (serum protein, A/G ratio, and bilirubin) were not altered following the administration of ^{109}Pd-protoporphyrin.

DISCUSSION

This work was designed primarily to demonstrate the usefulness of ^{109}Pd-protoporphyrin for homografting. Our initial work with (A) ^{63}Ni-protoporphyrin autoradiography and (B) lymphoid tissue histology following the administration of high-activity ^{109}Pd-protoporphyrin indicated that the lymphocytes present in the paracortical areas of the lymph nodes and in the spleen were critically affected by the irradiation.

It was our contention then that the destruction of mature competent lymphocytes in the spleen and lymph nodes would be followed by repopulation of these organs with immature immunoincompetent lymphoid cells originating in the bone marrow and thymus. Such traffic from bone marrow and thymus to the peripheral lymphoid tissue has been docu-

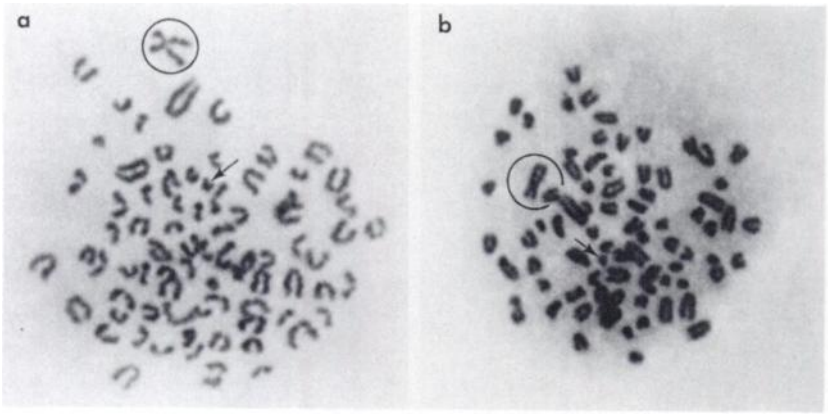

FIG. 5. (A) and (B) Male chromosome pattern in two cells obtained from male donor skin homografted onto female recipient treated with ^{109}Pd-protoporphyrin. Circled chromosome is X chromosome and arrow indicates Y chromosome.

TABLE 2. CYTOGENETIC ANALYSIS*

Cell source	Sex chromosomes	Chromosomes per cell <78	78	>78
Homograft (100 cells)	1 X	12	54	1
	2 X	2	25	1
	?	2	3	—
Male donor (50 cells)	1 X	14	74	2
	2 X	—	—	—
	?	4	6	—
Female recipient (50 cells)	1 X	4†	—	—
	2 X	12	76	—
	?	4	2	2

* Tabled entries are percentages of the total cells observed.

† Probably artifact due to random loss of chromosomes during slide preparation.

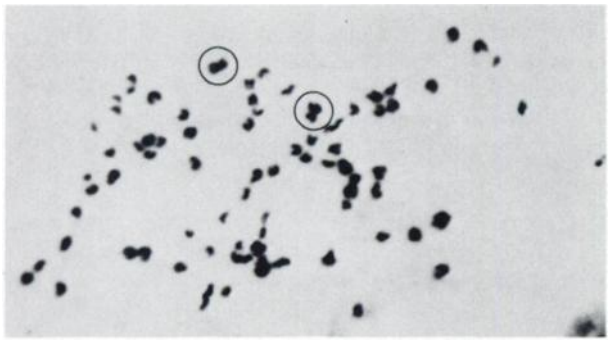

FIG. 6. Female chromosome pattern in representative skin cell obtained from recipient female dog at site near male homograft. Two X-chromosomes are circled.

mented (*21*). A homograft performed at this time should be tolerated in much the same manner as homograft tolerance induced prenatally after introduction of antigen into a functionally immature lymphoid system (*22*). Our contention that bone marrow is devoid of mature competent cells could be disputed in view of the graft versus host reaction encountered when bone marrow is infused into lethally irradiated animals (*23*). However, this is likely to be due in part at least to contamination of bone marrow with immunologically competent lymphocytes present in the circulation during bone marrow aspiration (*7*). That thymocytes are predominantly immature immunologically has been amply documented (*4*). In an experimental model approximating our own in design, a lethal dose of x-rays was administered to mice while the bone marrow and thymus were shielded. This permitted acceptance of skin homografts performed soon after irradiation (*24*). This result is strong evidence against the presence of committed lymphocytes in the bone marrow and thymus.

Our present criteria for homograft survival are: (A) absence of obvious acute rejection and continued healthy graft appearance, (B) retention of donor skin pigmentation, and (C) demonstration of donor (male) chromosomes within the graft site. In two dogs injected with 14 mCi/kg of ^{109}Pd-protoporphyrin i.v., the first two criteria are satisfied. The cytogenetic analysis performed on only one of these dogs satisfies the final criterion: it demonstrates male donor cells at the site of the homograft whereas the recipient is clearly female. The admixture in the homograft of two —X cells (28%) may be explained in either of two ways. The biopsy may have removed some female tissue from the graft–host juncture. Alternatively, as a consequence of slow rejection, invasive cells from the host may coexist with the grafted male cells. We cannot decide the issue on present evidence. We consider the former possibility most likely since the surgical procedure coupled with the uncertain dimensions of the graft almost certainly results in the excision of some host tissue.

In the experiments reported here, skin grafts were chosen as a test system both because of surgical simplicity and the fact that skin cells have strong antigenic expression [comparable in strength to leukocytes (*25*)]. On the basis of results reported here for skin homografts, we would anticipate similar results with other organ transplants.

The results reported here, although based on a small series of animals, are sufficiently promising to warrant further trials with ^{109}Pd-porphyrin complexes for control of homograft rejection.

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