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Received Oct. 14, 1968. P.S.E.B.M., 1969, Vol. 130.

Immunogenicity of Electrophoretically Purified Guinea Pig Transplantation Antigen (33651)

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Transplantation antigens are substances which induce specific accelerated (second-set) rejection of donor tissue grafts (1). Billingham *et al.* (1) first demonstrated hastened graft rejection following the administration of subcellular extracts. It is now generally accepted that subcellular membrane (2–5) and nonsedimentable (4, 6, 7) fractions can induce specific allograft immunity.

Recently there has been interest in solubilized transplantation antigens, i.e., in substances released from their usual location on the surface membrane of cells (8). Sonic energy was first employed to liberate transplantation antigen from spleen cells as a water-soluble material which was immunogenic when administered subcutaneously (6, 7). Recently, water-soluble transplantation antigen has been isolated from the spleen and lungs of inbred, histoincompatible guinea pigs and purified 10 thousandfold by discontinuous electrophoresis on polyacrylamide gels (9). Only one electrophoretic component (component 15, R_f 0.73–0.74) possessed antigenic activity as determined by its ability to elicit specific, delayed type hypersensitivity reactions in presensitized, allogeneic guinea pigs. This assay system was exceedingly rapid and sensitive, since 0.1 μg of antigen could be detected. Once the antigenic activity had been localized by this method, the immunogenicity tests described herein were performed to establish this material as a transplantation antigen. Only component 15 among the 17 electrophoretic fractions resolved on acrylamide gel was able to specifically sensitize allogeneic hosts against donor strain skin grafts.

Methods. Preparation of the antigen. Spleen and lungs obtained from 240 inbred, histocompatible strain 2 guinea pigs were minced in Tris–sucrose buffer (0.05 *M* Tris, 0.008 *M* magnesium chloride, 0.0025 *M* potassium chloride, 0.15 *M* sucrose, adjusted to pH 7.45, at 25°). Single cell suspensions were prepared by consecutive passes through nylon screening of 50 and 200 mesh. The suspension was centrifuged at 950*g* and the cell pellet obtained was suspended in Tris–sucrose buffer containing 3% (w/v) of acetic acid to lyse the erythrocytes. The residual cells were washed twice with Tris–sucrose buffer and then passed through a fine nylon mesh sieve. The cell concentration was adjusted to 30–40 $\times$ 10^6 cells/ml as determined on a model D Coulter counter at a threshold of 45/∞. Fifty-ml aliquots of the cell suspension were exposed to a 3-min burst of 15.5 W/cm^2 sonic energy generated over a diaphragm of 4.7 cm in a Raytheon model DF 101 magnostrictive oscillator. The sonication chamber was maintained at less than 5° by perfusion with alcohol at —10°. The sonicated suspension was first centrifuged at 3500 rpm, and then at 105,000–140,000*g* to remove the cellular membranes. The ultracentrifugal supernate was concentrated against Aquacide I, C Grade (Calbiochem) and then was passed in ascending fashion at a flow rate of 3–6 ml/hr through a column of Sephadex G-200 (2.5 $\times$ 100 cm) which had been equilibrated with Tris–glycine buffer,

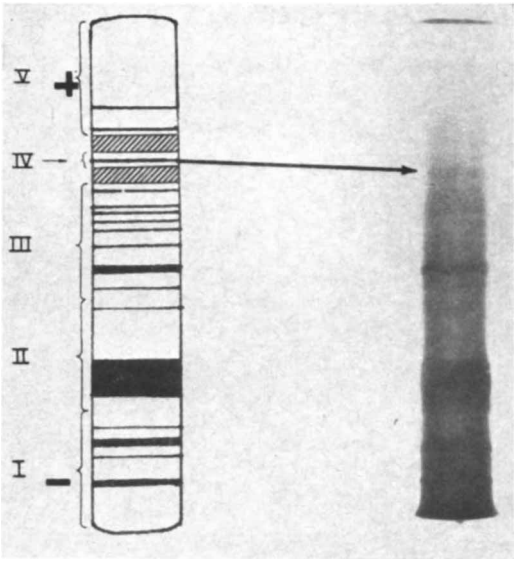

FIG. 1. Electrophoretic pattern of Strain 2 Sephadex fraction I on a 7.5% discontinuous polyacrylamide gel at pH 9.4. The gel was sectioned as indicated; the hatched areas on either side of component 15 were discarded.

pH 8.0 (0.5 *M* glycine, 0.2 *M* Tris, 0.5% mannitol). Fractions were collected in 3-ml aliquots and protein concentration was estimated by absorption at 280 mμ. The fraction (Sephadex fraction I) which appeared at the front of the inner volume was concentrated against 50% sucrose (enzymatic grade) and then further fractionated by electrophoresis in polyacrylamide gels.

Acyrlamide gel electrophoresis. Electrophoresis was performed at pH 9.4 essentially as described previously (9) but in the absence of urea in 7.5% acrylamide gels at 25° with a constant current of 2.5 mA per tube. When the bromphenol blue tracking dye had reached the end of the lower gel, electrophoresis was considered completed. The gels (4.5 cm in length) submitted to biologic assay were sectioned with a razor blade into 5 fragments as shown in Fig. 1.

Immunogenicity tests. Antigenic fractions prepared from strain 2 guinea pigs and purified by discontinuous polyacrylamide electrophoresis were administered to allogeneic strain 13 and to isogeneic strain 2 hosts following elution (48 hr at 4°) from acrylamide slices. The eluate of each set of gel slices was divided among six intradermal sites: 0.05 ml in each forefoot pad and 0.1 ml in each of four intradermal injection sites in the right thoracic region. The gel slices from which the eluate had been obtained were implanted subcutaneously on the same occasion in the right nuchal region.

Four days later, ear skin grafts from isogeneic strain 2 and allogeneic strain 13 animals were applied onto the right thorax of each animal according to the technique of Billingham and Medawar (10) but with graft edges overlapping the bed margins as recommended by Bauer (11). Plaster bandages were removed on the sixth postoperative day and the grafts were scored according to their gross appearance and then fixed in Bouin's solution. Semiserial microscopic sections (12) were independently coded and then scored

TABLE I. Immunogenicity of Strain 2 Acrylamide Fractions.

Antigen fraction[a]	2nd set rejection with strain 13 recipient and strain 2 donor[b]	2nd set rejection with strain 13 recipient and strain 13 donor[b]	2nd set rejection with strain 2 recipient and strain 13 donor[b]	2nd set rejection with strain 2 recipient and strain 2 donor[b]
None	0/12			
I	0/8	0/8	0/2	0/2
II	0/8	0/8	0/3	0/3
III	0/7	0/7	0/3	0/3
IV	8/9[c]	0/9	0/3	0/3
V	0/6	0/6	0/2	0/2
Origin	2/9	0/9	0/3	0/3

[a] Dose of antigenic material 1–3 μg for all fractions.

[b] Second-set rejection defined as greater than 60% destruction.

[c] Three of the eight second-set reactions were of the white graft type.

in double-blind fashion for the degree of epithelial survival by the criteria of Billingham *et al.* (13). In total, 138 sets of 20 slides each were scored randomly, thus maximizing objectivity. Greater than 60% epithelial destruction was considered evidence for an accelerated rejection response to the injected acrylamide elutate.

Results. Twenty spleen equivalents of strain 2 polyacrylamide fractions I, II, III, IV, or V were administered to allogeneic strain 13 and to isogeneic strain 2 guinea pigs. The fate of challenge grafts applied onto strain 2 hosts isogeneic with the antigen donors was not affected by any of the acrylamide fractions. Thus, no wound healing inhibitory factor would be demonstrated as reported by Billingham (14) with bacterial extracts. On the other hand, administration of fraction IV (component 15) to allogeneic strain 13 hosts yielded accelerated rejection of 8 out of 9 test strain 2 grafts but none of the 9 isogeneic strain 13 grafts were affected (Table I). Three of the strain 2 challenge grafts were destroyed in white graft fashion, i.e., before they had established vascular connections with the host bed, while the remaining 6 grafts exhibited 75, 40, 40, 25, 25, and 10% epithelial survival. None of the strain 13 grafts applied onto the same strain 13 hosts at the time of challenge with strain 2 skin grafts evidenced less than 90% survival. Grafts applied after the administration of acrylamide fractions I, II, III, or V yielded never less than 60% epithelial survival and 82% of the test strain 2 grafts never had greater than 75% epithelial survival. It is of interest that administration of the material located at the origin of the acrylamide gel yielded two cases of specific accelerated rejection, presumably due to aggregated fraction IV which failed to enter the acrylamide gel.

Figure 2A shows the histologic appearance at 5 days of a strain 13 skin graft applied onto a strain 2 host which had been sensitized with strain 2 fraction IV. The hyperplastic, acanthotic, healthy epithelium and well vascularized dermal field of this graft signifies a first-set reaction (see also Bauer (11), Fig. 3a). In contrast, the strain 2 grafts (Fig. 2B, C, and D) applied onto strain 13 hosts presensitized with strain 2 fraction IV revealed a thinned, necrotic epithelium and a poorly vascularized acidophilia, inflamed dermis, stigmata of strong second-set reactions (see also Bauer (11), Fig. 4d).

Discussion. Twenty spleen equivalents, i.e., 1–3 μg, of electrophoretically purified fraction IV induced a specific state of immunity, such that donor-type allogeneic but not isogeneic skin transplants were rejected as second-set grafts. Furthermore, this material did not sensitize isogeneic donor strain animals to reject either isogeneic or allogeneic grafts in accelerated fashion.

It has been suggested that solubilization of transplantation antigens from their sites on cell membrane surfaces results in the loss of lipoidal attachments necessary for immunogenicity. Therefore, soluble antigens would be expected to be immunogenic only when combined with an appropriate adjuvant (15). However, the bulk of the evidence regarding the induction of immunity against transplantation antigens suggests that the route, method, and schedule of antigen administration (16) are more important than the source or physical form of the antigenic materials (17). Although Freund's adjuvant has a crucial role in the induction of delayed-type hypersensitivity against a variety of antigens (18), its role in transplantation immunity is uncertain. Numerous studies (19–21) employing subcellular antigens without and with complete Freund's adjuvant failed to reveal significant enhancement of the allograft response by this reagent. However, these studies are not conclusive: the transplantation antigens were employed in a crude state, although in some instances relatively lipid-free, and it is possible that contaminant or aggregated materials in these preparations may have functioned as adjuvants. Since the purified guinea pig antigens employed herein are relatively chemically homogeneous, it was decided for reasons of economy to administer them simultaneously in soluble form and entrapped within polyacrylamide gel, which is recognized as an excellent adjuvant (22).

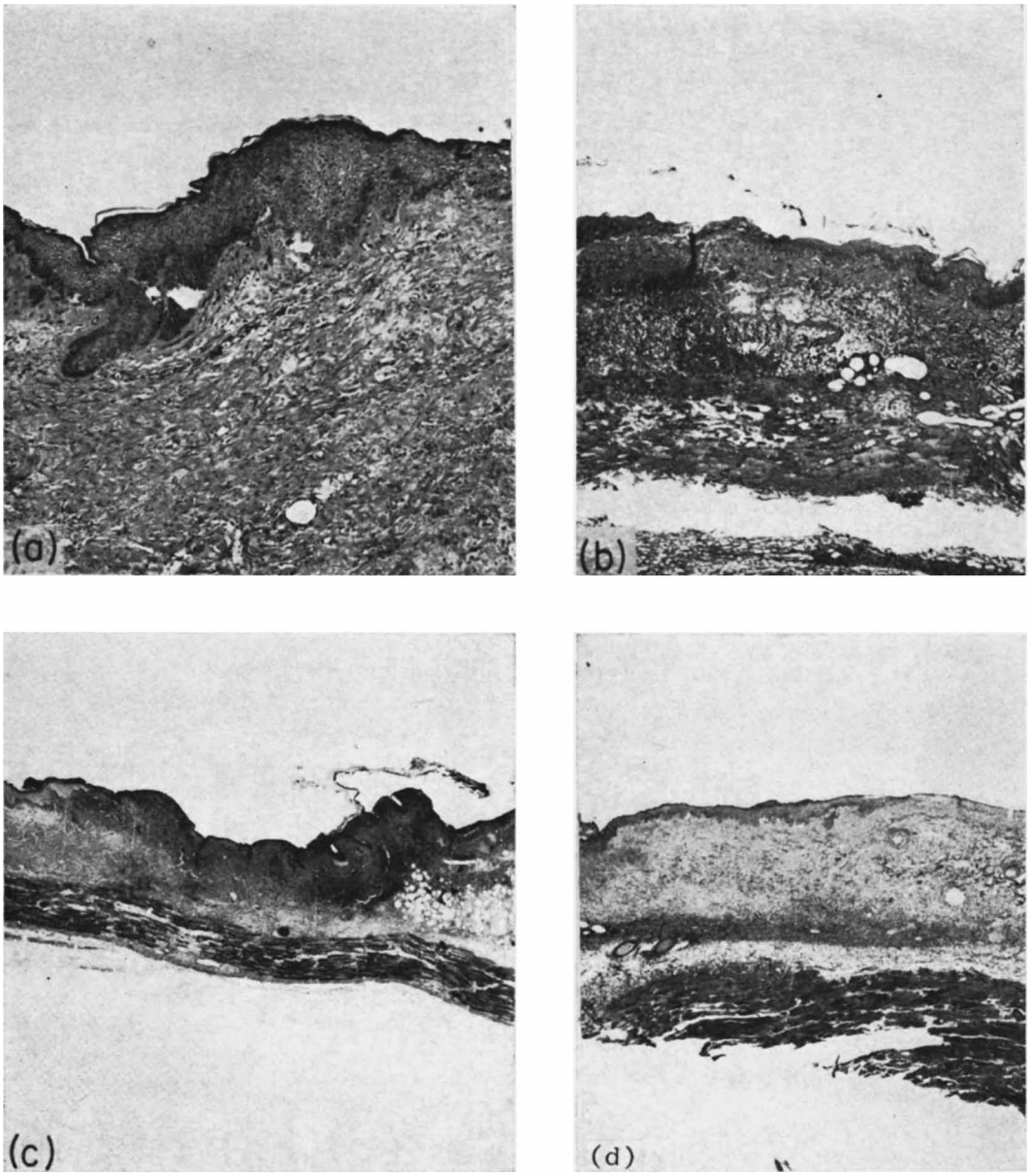

FIG. 2. Histologic appearances of skin grafts on hosts which received strain 2 acrylamide component 15. Stained with H & E. (A) Strain 13 graft applied onto a Strain 2 host: first-set graft appearance ×65. (B,C,D) Strain 2 grafts applied onto strain 13 hosts: second-set appearances: ×16; ×12; ×16.

Further studies on the immunogenicity of this material administered (i) in soluble monomeric form (23), (ii) in soluble polymeric form, and (iii) in Freund's adjuvant or polyacrylamide gel may clarify the nature of and the requirements for an immune response to purified transplantation antigens.

The immunogenic properties of acrylamide fraction IV (component 15) presented herein, as well as the previous demonstration that this material elicits specific cutaneous hypersensitivity responses in sensitized guinea pigs (9), prove that this purified component is a transplantation antigen, i.e., a substance

with an immunologically specific effect on the fate of test grafts.

Summary. Water-soluble fraction IV (component 15; R_f 0.73–0.74) purified by electrophoresis of sonicated extracts of strain 2 guinea pig spleen cells, was shown to induce specific allograft immunity in inbred strain 13 animals, thus establishing this material as a transplantation antigen, i.e., a substance which specifically effects the fate of grafts.

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Received Oct. 15, 1968. P.S.E.B.M., 1969, Vol. 130.