Proc. Natl. Acad. Sci. U. S. **47**, 105 (1961).

2. Pasternak, G. and Pasternak, L., J. Natl. Cancer Inst. **38**, 157 (1967).

3. Hartley, J. W., Rowe, W. P., Capps, W. I., and Huebner, R. J., in press.

4. Mims, C. A., J. Pathol. Bacteriol. **91**, 395 (1966).

5. Mims, C. A. and Subrahmanyan, T. P., J. Pathol. Bacteriol. **91**, 403 (1966).

6. Rawls, W. E. and Melnick, J. L., J. Exptl. Med. **123**, 795 (1966).

7. Hopps, H. E., Bernheim, E. L., Nisalak, A., Tjio, J. H., and Smadel, J. E., J. Immunol. **91**, 416 (1963).

8. Melnick J. L., Wenner, H. A., and Rosen, L., *in* "Diagnostic Procedures for Viral and Rickettsial Diseases" (E. H. Lennette and N. J. Schmidt, eds.), 3rd ed., p. 194. Am. Public Health, New York (1964).

9. Vaheri, A., Sedwick, W. D., Plotkin, S. A., and Maes, R., Virology **27**, 239 (1965).

10. Rawls, W. E., Desmyter, J., and Melnick, J. L., Proc. Soc. Exptl. Biol. Med. **124**, 167 (1967).

11. Marcus, P. L. and Carver, D. H., Science **149**, 983 (1965).

12. Parkman, P. D., Buescher, E. L., Artenstein, M. S., McCowan, J. M., Mundon, F. K., and Druzd, A. D., J. Immunol. **93**, 595 (1964).

13. Desmyter, J., Rawls, W. E., Melnick, J. L., Yow, M. D., and Barrett, F. F., J. Immunol. **99**, 771 (1967).

14. Rawls, W. E., Desmyter, J., and Melnick, J. L., J. Am. Med. Assoc. **203**, 627 (1968).

15. Rawls, W. E., Progr. Med. Virol. **10**, 238 (1968).

16. Plotkin, S. A., Boue, A., and Boue, J. G., Am. J. Epidemiol. **81**, 71 (1965).

17. Maassab, H. F. and Veronelli, J. A., J. Bacteriol. **91**, 436 (1966).

Received June 11, 1968. P.S.E.B.M., 1968, Vol. 129.

Cytotoxic Antibody Response to Skin Allografts in Calves: Effects of Extracorporeal Irradiation of Lymph* (33349)

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The immunological nature of skin allograft rejection has been firmly established. The mechanism responsible for this process, however, has not been fully resolved. Many investigators favor the view that rejection is based principally upon the action of sensitized mononuclear cells (1, 2). On the other hand, there are those who feel that humoral antibodies play a major role by exercising a primary cytotoxic action on the graft (3). Accumulating evidence for the participation of complement in the rejection of skin allografts (4, 5) indirectly supports the role of complement-dependent antibodies in the effector mechanism. Lymphocytotoxic antibodies (6) have been demonstrated in the sera of animals (7–9) and man (10) following skin allografting, and there is increasing evidence that these antibodies are directed against histocompatibility antigens (11).

* Research supported by the United States Atomic Energy Commission.

Previous studies from this laboratory have shown that extracorporeal irradiation of thoracic duct lymph (ECIL) will prolong the survival time of skin allografts in calves (12). Furthermore, when the skin grafts were placed within the drainage bed of the thoracic duct (posterior grafts), the grafts remained intact until ECIL was discontinued (13). The reason proposed for the survival of posterior skin grafts was that immunologically activated lymphocytes emerging from the regional lymph nodes were destroyed by ECIL prior to entry into the blood. In contrast, if the grafts were not within the drainage bed (anterior grafts), ECIL only delayed the graft rejection time.

Theoretically, ECIL should not interfere with the production or release of antibody from the lymph nodes draining posterior allografts, and certainly the amount of irradiation received by the antibody molecules would not alter their activity (14). The object of the present experiments was to exam-

ine the cytotoxic antibody response in instances where the rejection of skin allografts had been prolonged by ECIL. The results indicate that antibody response, like graft rejection, can under certain conditions be markedly influenced by ECIL.

Materials and Methods. Twenty-nine calves weighing 80–200 kg were used in this study. A thoracic duct-venous shunt was prepared in 11 calves, as previously described (15). Lymph was collected continuously into sterile plastic bags kept at 5°. From the bag the lymph was pumped through a silicone rubber coil surrounding a gamma irradiation source (^{137}cesium) and back into the jugular vein. The dose of radiation received by 80–90% of the cells or molecules in transit through the irradiation field was 342-1000 rad; the remaining 10–20% received up to 37,000 rad. The collection system was heparinized to prevent clotting.

On the day ECIL was begun 6–8 full thickness allografts, about 1 cm in diameter, were removed from the dorsum of the ear of the donor and transplanted either in the area of the iliac crest (posterior grafts) or on the right side of the withers (anterior grafts). Compression dressings were removed on the sixth day after grafting and the grafts were inspected daily thereafter.

Cytotoxic antibody assays were based on the method of Gorer (6). One-tenth ml of medium 199 containing 1×10^6 donor blood lymphocytes, 0.1 ml of recipient serum, and 0.1 ml of guinea pig serum were mixed in plastic culture tubes (12 × 75 mm). After 30-min incubation at 38.5°, 0.2 ml of trypan blue in saline (1:750) was added and the percentage of stained cells determined by counting 200 cells in a hemocytometer. All recipient sera was diluted 1:4 with medium 199. When cytotoxicity could not be detected, even following graft rejection, rabbit serum was used as a source of complement to increase the sensitivity of the assay (10). Complement, cell, and normal sera controls were incorporated into each assay.

Results. Six untreated calves served as controls. Allograft survival in this group was 9–11 days. The cytotoxic antibody responses are shown in Fig. 1. Cytotoxicity was first

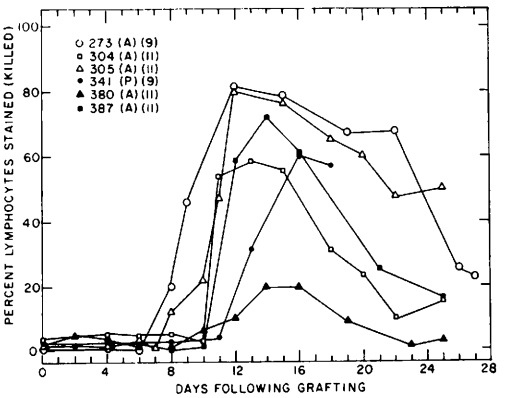

FIG. 1. Cytotoxic antibody response of untreated calves: Assay performed with guinea pig complement; P = posterior grafts; A = anterior grafts; day of rejection in parentheses.

detected in the serum 8–12 days after grafting. It reached a peak shortly after graft rejection and fell rapidly thereafter.

Eleven calves received continuous ECIL beginning on the day of allografting. Two calves received anterior grafts and 9 received posterior grafts. ECIL resulted in a 85–90% depression in thoracic duct lymphocyte output and a 60–70% reduction in the blood lymphocyte count. The anterior grafts of calves 284 and 295 were rejected on days 15 and 16. Due to a technical problem, calf 295 received ECIL for only 11 days. Of the 9 calves with posterior grafts, 6 rejected while ECIL was continuing (days 10–18). The cytotoxic antibody responses of these 6 calves are shown in Fig. 2 along with calves 284 and 295. With one exception (calf 344), there was a slight delay in the appearance of cytotoxicity in the serum; however, the level of activity reached was not markedly different from that seen in untreated calves.

The posterior grafts of 3 calves remained intact until after ECIL was discontinued (Fig. 3). Grafts were allowed to be rejected in calves 257 and 296. The grafts of 325, however, were surgically excised on the day ECIL was stopped. When the assay included guinea pig serum, no significant amount of cytotoxicity was detected in the sera of these 3 calves, even at the time of rejection. With the addition of rabbit serum as a source of complement, definite responses were observed, as shown in Fig. 3. Cytotoxicity was

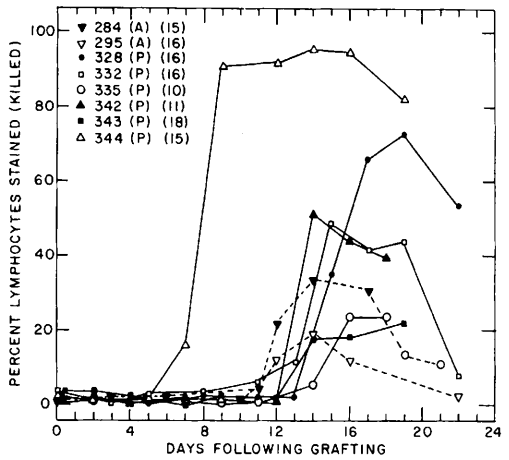

FIG. 2. Cytotoxic antibody response of calves which received continuous ECIL beginning on the day of grafting. All allografts were rejected during ECIL. Assay performed with guinea pig complement; P = posterior grafts; A = anterior grafts; day of rejection in parentheses.

first detected in the sera of calf 257 between days 16–18. Following the termination of ECIL (day 21) the activity quickly increased, reaching a peak about the time of graft rejection. Cytotoxic antibody was found in the lymph prior to its detection in sera and the level remained low even after ECIL was discontinued. The response in calf 296 was similar to 257 with the exception of an 8–10 day interval between termination of ECIL (day 28) and peak serum activity. Grafts were rejected on day 9 post-ECIL.

No cytotoxicity was detected in the serum of calf 325 for 26 days. The ECIL was then discontinued and the grafts were removed surgically. Within 2-4 days activity was detectable. Calf 325 was regrafted from the same donor on day 35 and a typical secondary antibody response followed.

Discussion. The results of these experiments can best be explained on the premise that the immune response is amplified and propagated throughout the body by stimulated lymphoid cells which originate in the regional lymph nodes and enter the circulation by way of the efferent lymph (16). Efferent lymph from the posterior part of the body enters the blood via the thoracic duct. Stimulated lymphocytes emerging from nodes draining posterior skin allografts would, therefore, be destroyed by ECIL.

Experimentally, ECIL markedly depressed the cytotoxic antibody response in the 3 calves which maintained posterior allografts for the duration of treatment. When ECIL was discontinued, cytotoxicity of the sera increased; presumably the result of stimulated, viable lymphocytes entering the circulation via the thoracic duct. Surgical removal of grafts indicated that the process of rejection was not necessary for the rise in activity. The post-ECIL antibody response of these 3 calves was not of the magnitude seen in untreated claves, but this could be the result of fewer stimulated cells being released into the efferent lymph at the time ECIL was terminated (days 21–28). Hall (17) reported that in efferent lymph of nodes draining skin allografts, the number of basophilic cells had decreased markedly by the 20th postgraft day. Apparently very few stimulated lymphocytes are needed for graft rejection.

The low level of cytotoxicity found in the sera of calves 257 and 296 prior to the termination of ECIL was, in all probability, due to antibody synthesis in the regional lymph nodes. The presence of cytotoxic antibody in thoracic duct lymph prior to detection in the serum would support this concept. Based on the sensitivity of the assay method used, these experiments suggest that the antibody synthesized in the regional nodes is only a minor part of the total antibody synthesized in response to allografts in an untreated animal. Apparently antigen(s) do not bypass

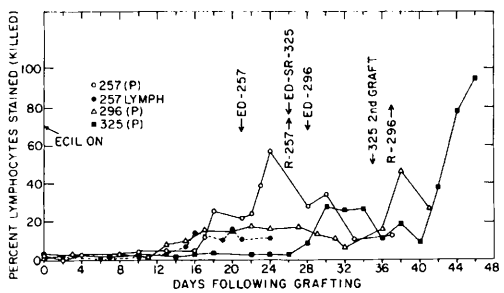

FIG. 3. Cytotoxic antibody response of calves in which posterior skin allografts remained inact until ECIL was discontinued. Assay performed with rabbit complement; R = rejection; SR = surgical removal; ED = ECIL discontinued.

the regional nodes in suffiicent quantity to initiate an immune response elsewhere.

Failure of ECIL to suppress the cytotoxic antibody response and maintain posterior skin grafts in 6 calves was somewhat surprising, in view of previously published results (13). The most likely explanation for this failure is that lymphatic–venous communications existed posterior to the site of cannulation and, therefore, only a portion of the stimulated lymphocytes were destroyed by ECIL. It might also be argued that the successful repression of graft rejection and antibody response in some calves was due to a close genetic relationship between the donor and recipient. This seems unlikely, however, since grafts were rejected shortly after the termination of ECIL. Unpublished data using chimeric twin calves also argues against a close genetic relationship. The slight delay in onset of cytotoxicity and graft rejection seen in these 6 calves, and the 2 calves with anterior grafts, was presumably due to a partial depletion of immunologically competent and/or stimulated lymphoctes as a result of ECIL.

It is impossible to define the role of humoral antibody in skin allograft rejection from these experiments. Grafts remained intact for many days (calf 296) in the presence of detectable cytotoxicity. When compared to untreated calves, antibody levels were very low in calves rejecting grafts following the termination of ECIL. There appeared to be no relationship between the onset or level of cytotoxicity and graft rejection. Indeed, the results would seem to favor lymphoid cells as the prime mediators of allograft rejection.

Summary. The cytotoxic antibody response to skin allografts was examined in calves which received continuous ECIL for up to 28 days. In 3 calves, which maintained posterior skin grafts until ECIL was discontinued, the antibody response was markedly depressed. Following the termination of ECIL cytotoxicity of the sera increased. It was concluded that the antibody response is propagated throughout the body by radiosensitive cells entering the blood via efferent lymphatics.

1. Medawar, P. B., Proc. Roy. Soc. (London), Ser. B. **149**, 145 (1958).

2. Billingham, R. E., Transplantation **5**, 976 (1967).

3. Stetson, C. A., Advan. Immunol. **3**, 97 (1963).

4. Coppola, E. D. and Villegas, G. R., Proc. Soc. Exptl. Biol. Med. **125**, 1071 (1967).

5. Volk, H., Mauerberger, D., Rother, K., and Rother, U., Ann. N. Y. Acad. Sci. **120**, 26 (1964).

6. Gorer, P. A. and O'Gorman, P., Transplant. Bull. **3**, 142 (1956).

7. Terasaki, P, I., Proc. Soc. Exptl. Biol. Med. **106**, 133 (1961).

8. Altman, B. and Simonsen, M., Ann. N. Y. Acad. Sci. **120**, 28 (1964).

9. Ballantyne, D. L. and Stetson, C. A., Ann. N. Y. Acad. Sci. **120**, 7 (1964).

10. Walford, R. L., Gallagher, R., and Troup, G. M., Transplantation **3**, 387 (1965).

11. Vredevoe, D. L., Mickey, M. R., Goyette, D. R., and Terasaki, P. I., Ann. N. Y. Acad. Sci. **129**, 521 (1966).

12. Chanana, A. D., Brecher, G., Cronkite, E. P., Joel, D. D., and Schnappauf, H. P., Radiation Res. **27**, 330 (1966).

13. Joel, D. D., Chanana, A. D., Cronkite, E. P., and Schiffer, L. M., Transplantation **5**, 1192 (1967).

14. Rosenau, W. and Moon, H. D., J. Immunol. **96**, 80 (1966).

15. Chanana, A. D. and Cronkite, E. P., Am. J. Vet. Res. **27**, 683 (1966).

16. Hall, J. G., Morris, B., Moreno, G. D., and Bessis, M. C., J. Exptl. Med. **125**, 91 (1967).

17. Hall, J. G., J. Exptl. Med. **125**, 737 (1967),

Received June 14, 1968. P.S.E.B.M., 1968, Vol. 129.