FETAL RESPONSE TO ANTIGENIC STIMULUS

IV. Rejection of Skin Homografts by the Fetal Lamb*

By ARTHUR M. SILVERSTEIN, Ph.D., ROBERT A. PRENDERGAST,‡ M.D.,
Captain, Medical Corps, United States Army Reserve,
and KEITH L. KRANER,
Captain, United States Air Force, Veterinary Corps
(*From the Immunobiology Branch, Armed Forces Institute of Pathology, Washington, D. C.*)

(Received for publication, March 9, 1964)

Recent evidence has indicated that the ability to develop an immunologic response to antigenic stimulus first appears during the fetal stage in certain species (1–4). Earlier papers in this series (5, 6) have indicated that the fetal lamb *in utero* is able to form antibodies as early as the first half of gestation. Curiously, the time of maturation of the immune response in these fetuses seems to be a function of the antigen employed. While bacteriophage ϕX 174 stimulates antibody formation before the 53rd day of gestation, horse ferritin does not assume antigenicity until about the 65th day, nor does ovalbumin until about the 125th day. Substances such as diphtheria toxoid and *Salmonella typhosa* were found to be ineffective throughout fetal life as elicitors of an antibody response.

Schinkel and Ferguson (7) reported that the fetal lamb is able to reject allogeneic skin grafts specifically at about the 117th day of gestation. This study of rejection of orthotopic skin grafts in the fetal lamb was undertaken (*a*) to confirm the observations of Schinkel and Ferguson; (*b*) to determine the earliest fetal age at which allogeneic graft rejection would occur; (*c*) to study the rate of graft rejection in the young fetus; (*d*) to examine the effect on graft rejection of various donor-recipient relationships; (*e*) to evaluate the morphologic and serologic changes accompanying the rejection process; and (*f*) to clarify the role of circulating antibody in the mechanism of graft rejection. The latter investigation, a preliminary report of which has appeared elsewhere (8), is made possible by the unique situation that exists in the ovine fetus, which receives no maternal γ-globulin across the placenta and synthesizes little or none itself (6, 9).

* This investigation was supported in part by a research contract, Project No. 6X61-01-001-03, from the Medical Research and Development Command, United States Army, Washington, D. C.

‡ Present address: The Rockefeller Institute.

Downloaded from http://rup.silverchair.com/jem/article-pdf/119/6/955/1081501/955.pdf by guest on 05 February 2023

Materials and Methods[1]

Fetal Lambs.—Through the courtesy of the Sheep and Fur Animal Research Branch, Animal Husbandry Research Division, United States Department of Agriculture, Beltsville, Maryland, ewes of randomly mixed breeds were bred under controlled conditions permitting the date of conception to be established within 1 to 2 days. The gestation period of the sheep is 150 days.

Source of Skin Grafts.—In order to test as many as possible of the parameters of the rejection process, skin grafts were employed from both related and unrelated sheep. These included split thickness grafts from the mother, from unrelated adult ewes, and from unrelated newborn lambs, as well as full thickness grafts from unrelated fetuses. In addition to these fresh skin grafts, a number of procedures were performed with frozen fetal skin, prepared according to the method of Billingham (10).

Since these grafts were to be placed on fetuses *in utero*, great care was exercised in the cleansing of the donor skin prior to excision of the graft, to avoid gross contamination and fetal infection. In certain experiments on the cellular and protein responses accompanying graft rejection, it was desired to exclude completely all exogenous antigens save for those histocompatibility antigens of the donor skin itself. To this end, donor skin was removed from fetuses of an appropriate age that had been delivered by Caesarian section with the exercise of rigorously aseptic technic. These grafts were immediately transferred to the recipient fetuses. The data on the grafts in Table I include the age and origin of each of the grafts employed.

Surgical Procedure for Fetal Grafting.—During the early phases of this study, the surgical approach described in our earlier report (5) combined with the technic of Schinkel and Ferguson (7) was employed. As experience was gained, a number of useful modifications were introduced, resulting in a relatively simple and rapid procedure by which fitted skin homografts and autografts could be applied successfully to fetal lambs of varying age with little likelihood of interrupting pregnancy. Its principal points involve: (*a*) use of fluothane general anesthesia; (*b*) inclusion of the allantois in the incision through the fetal membranes, since this seemed to assist in healing; (*c*) exteriorization of the entire uterine horn, incising a window through which to work on large fetuses and bringing the smaller ones (less than 15 to 20 cm in length) completely out of the uterus; (*d*) application of fitted grafts sutured with fine silk, rather than the buried grafts favored by Schinkel and Ferguson; and (*e*) closure of the fetal membranes with a silk purse string suture.

The grafts were generally between 1 and 2 cm^2 in size. In many instances two grafts were applied to the same fetus, some of which were autografts serving as controls of technic. This involved the lifting of a square of skin and its replacement after rotation of 180°. In other cases, a maternal graft or skin from an unrelated adult was applied in conjunction with fetal skin to detect possible differences in rejection rates.

Delivery of Fetuses and Collection of Specimens.—At an appropriate interval after grafting, the fetus was delivered by hysterotomy and bled from the umbilical cord as outlined previously (5). In some instances, specimens were taken from ungrafted twins for control purposes. The fetuses were autopsied immediately after delivery. In each case, the grafts and surrounding normal skin were sampled, as well as draining and distant lymph nodes, spleen, thymus, and other tissues. A portion of each tissue was fixed in Carnoy's fixative, and the remainder in buffered formalin.

Examination of Specimens.—Judgment of the primary acceptance of the various grafts and of their ultimate acceptance or rejection was made by histopathologic examination. In addition, the graft beds and draining lymph nodes were carefully examined for the presence

[1] The "Principles of Laboratory Animal Care" as promulgated by the National Society for Medical Research were observed during this investigation.

Downloaded from http://rup.silverchair.com/jem/article-pdf/119/6/955/1081501/955.pdf by guest on 05 February 2023

of mature or immature plasma cells that might indicate the formation of antibody as a component of the rejection process. In all instances, sections stained with both hematoxylin and eosin and with methyl green–pyronin were examined.

Immunoelectrophoretic analyses of the cord bleedings were performed as described previously (6) in order to determine whether detectable immunoglobulin production accompanied the response to homografts. These patterns were developed with hyperimmune rabbit anti-adult sheep antiserum, chosen to contain appreciable amounts of antibody against the γ-, β_{2m}-, and β_{2A}-globulins. A number of lymph nodes and spleens from grafted animals were cultured in the presence of C^{14}-labeled amino acids, as in earlier studies (6). Immunoelectrophoresis and autoradiography of the tissue culture supernatants were then performed to detect with greater sensitivity the synthesis of labeled proteins by these tissues. We are indebted to Dr. G. J. Thorbecke for her kind assistance with these preparations.

RESULTS

During the course of the studies described in this report, grafting procedures involving penetration of the uterus and fetal membranes were performed upon 20 fetal lambs. One ewe had an anesthetic death immediately following the procedure, and in another instance a fetus was later delivered dead, apparently the result of trauma accompanying the original operation. In one case, both homograft and autograft were technically unsuccessful. Seventeen fetuses survived to furnish useful data, which are summarized in Table I, together with the appropriate information on fetal ages, the source of the grafted skin, its stage of rejection, and the nature of accompanying response in the recipient.

Graft Rejection and Fetal Age.—Four fetuses between 64 and 67 days of gestation were grafted with fitted skin grafts from adult (split thickness) and fetal (full thickness) sources. The animals were delivered at intervals between 9 and 21 days after grafting, and the grafts and draining lymph nodes were examined. In no case was a clear-cut rejection process visible (Table I). The grafts had taken well and resembled autografts, even after 21 days. Only one graft (lamb 35-61, adult homograft) had a mild, diffuse mononuclear infiltrate in the graft bed, which did not appear to involve interference with graft vitality, perhaps because of exogenous contaminants introduced with the graft. The draining lymph nodes at this early fetal age were found to be similar to those seen in ungrafted animals of the same age, again indicating that the fetuses had not responded in any significant manner to homografts applied at this stage of gestation.

A number of other fetal lambs were grafted between the 77th and the 139th day of gestation with a variety of fitted skin grafts from different sources. The grafts were examined between 7 and 15 days later, about half being checked on the 10th day (Table I). In all but one case, the autografts applied for control purposes had taken well and were undisturbed by any host reaction. In contrast, every homograft applied during this interval was found to be involved in an active and typical rejection process. Even the grafts examined at 7 or 8 days showed unequivocal evidence of rejection, indicating that the process had

Downloaded from http://rup.silverchair.com/jem/article-pdf/119/6/955/1081501/955.pdf by guest on 05 February 2023

TABLE I

Response to Orthotopic Skin Homografts in Fetal and Newborn Lambs

Lamb No.	Gestation age at grafting	Interval before graft removal	Source of graft	Stage of rejection	Remarks
	days	*days*			
35-61	64	9	Frozen fetal	No rejection	
			Adult	Very early (?)	
35-71	65	14	Frozen fetal	No rejection	
			Adult	No rejection	
31-21	65	15	Maternal	No rejection	
30-41	67	21	Frozen fetal	No rejection	
			Adult	No rejection	
30-52	77	11	Frozen fetal	Late rejection	
34-31	82	12	Maternal	Late	
			Autograft	No rejection	
35-01	82	15	Frozen fetal	Late	
			Autograft	No rejection	
34-41	97	9	Maternal	Late	
			Autograft	No rejection	
32-31	122	14	Fresh fetal	Very late	
34-91	120	10	Fresh fetal	Late	Rabbit anti-globulins*
34-21	122	10	Fresh fetal	Late	Rabbit anti-globulins
33-21	122	10	Fresh fetal	Middle	Normal rabbit serum
31-91	125	10	Newborn lamb	Late	Rabbit anti-globulins
			Autograft	No rejection	
35-51	125	10	Fresh fetal	Middle	Normal rabbit serum
31-12	130	8	Fresh fetal	Late	Rabbit anti-globulins
30-71	136	7	Fresh fetal	Late	
30-92	139	10	Fresh fetal	Very late	
35-91	Day of birth‡	7	Newborn	Middle	
		10	Newborn	Late	
35-92	Day of birth	7	Newborn	Middle-late	
		10	Newborn	Late	
34-51	Day of birth	7	Newborn	Middle-late	
		10	Newborn	Late	
34-52	Day of birth	7	Newborn	Middle-late	
		10	Newborn	Late	
34-11	Day of birth	10	Newborn	Middle-late	

* After grafting, lambs were injected with 20 ml of whole rabbit anti-sheep β_{2m}- and γ-globulins, or with 20 ml of normal rabbit serum.

‡ Gestation period in sheep is about 150 days.

Downloaded from http://rup.silverchair.com/jem/article-pdf/119/6/955/1081501/955.pdf by guest on 05 February 2023

commenced at least 1 to 2 days earlier. By the 10th to the 14th day, the grafts were obviously at a very late stage of rejection. In all instances, the typical reaction in the draining lymph nodes confirmed the nature and stage of the rejection process.

Finally, newborn lambs were grafted by exchanging full thickness skin among five animals on the day of birth. In each instance, two grafts were applied on a recipient from the same donor, so that one could be taken at 7 and the other at 10 days. Examination of these tissues showed that the rejection process in the newborn lamb appeared to be no different from that occurring in the competent fetal animal. The grafts were accepted in a typical fashion, and at 7 days active rejection was evident. By the 10th day, each of the grafts was in the final stages of rejection, with vascular stasis and accompanying necrosis.

Origin of the Donor Skin.—A variety of skin homografts was employed to ascertain whether the origin of the graft, its age, and the donor-recipient relationship would affect graft survival. Fresh and frozen full thickness fetal skin, split thickness newborn skin, and split thickness skin from maternal and from unrelated adult sheep were employed. In no instance did the origin of the skin or its manner of preparation appear to influence the outcome of the recipient's response, either temporally or quantitatively. All homografts applied after the onset of competence in the recipient were rejected with equal efficiency and dispatch.

Morphology of Fetal Graft Rejection.—Initial healing of grafts was similar morphologically to that described for other species (11). In those fetuses sacrificed early after implantation of the graft there was primary union between donor and host epithelial margins, regardless of the type of graft,—whether of maternal, unrelated adult, or fresh or frozen fetal skin. In the case of adult tissues new hair growth was observed in a few instances. Cross-section of the graft also demonstrated firm union and the establishment of vascular anastomoses between graft and recipient bed in these early cases. Histologic demonstration of this primary take was also expressed in the survival and hypertrophy of epithelium and in the presence within the graft of patent blood vessels containing formed elements. At this stage the homografts resembled autografts on animals of similar gestational age. In both instances, a few mononuclear and polymorphonuclear cells were noted in the graft bed. In maternal and unrelated adult grafts, hypertrophic follicular epithelium was also present.

The rejection of grafts, once begun, followed the pattern previously described for acute graft rejection (11). In all instances in which adult tissue was used, whether maternal or unrelated, rejection was preceded by grossly discernible edema of the graft followed by hemorrhage and later retraction of the graft bed. Vascular stasis and hemorrhage were readily apparent histologically within the graft and the adjacent graft bed. The mononuclear infiltrate was most prominent about small vessels in the graft bed and adjacent to disintegrating

Downloaded from http://rup.silverchair.com/jem/article-pdf/119/6/955/1081501/955.pdf by guest on 05 February 2023

epithelium, especially at the junction of graft and host tissue. These cells ranged from small lymphocytes to larger mononuclear forms with indented nuclei and a larger amount of cytoplasm. No evidence of formation of typical adult plasma cells was noted in the graft or graft bed, nor was pyroninophilia seen in the mononuclear cells. Varying degrees of polymorphonuclear infiltrate were present, most apparent in the later stages of rejection. Epithelial degeneration was marked at this time (after 7 days) in those animals on which the graft was placed on or after the 80th day of gestation. One curious feature, perhaps a result of the intrauterine milieu, was the apparent survival of a thin outer rim of epithelium in some cases in which the remainder of the graft had become completely non-viable. It may have been that the superficial cells of the graft, bathed in amniotic fluid, were able to retain some degree of viability even though the circulation had become completely stagnant.

Grafts of fresh and frozen fetal skin behaved in a similar fashion to adult grafts, except that epithelial proliferation about hair follicles was not so apparent. Examination of the frozen grafts early in the course of rejection showed a slough of the outer margin of epithelium, though follicular and basal layers were well preserved. It is probable that the preservation of this tissue was only partial and that the most peripheral layers of epithelium were not viable after transfer.

Morphology of the Lymph Node Response.—Normal, non-stimulated lymph nodes in fetal lambs at 60 to 90 days' gestational age were characterized by a preponderance of medullary channels lined by endothelium. The rather thin medullary cords at this time contained reticulum and other non-descript mesenchymal cells, few lymphocytes, and varying degrees of extramedullary hematopoiesis. The cortex was poorly developed, and no good corticomedullary demarcation was noted. In later gestation the cortex contained more lymphoid elements and occasional small primary lymphatic follicles. Hematopoiesis in the medullary cords was less marked, and the cords themselves contained more lymphoid cells and were perceptibly thicker. At term, the lymph node showed a well defined and cellular cortex. At no time were plasma cells noted in unstimulated animals. In addition, there was only the most minimal diffuse pyroninophilia in the cytoplasm of cells within the primitive follicles of the cortex.

The lymph nodes draining the homografts in the process of rejection showed an altered morphologic picture. In contrast to the normal, poorly developed cortex, these nodes exhibited "geographic" areas of increased cortical development with many more lymphocytes of all sizes. In the early stages, the most striking change was a proliferation of large lymphoid cells with pyroninophilic cytoplasm, large vesicular nuclei, and prominent nucleoli. These cells were scattered throughout the active zone of the cortex and adjacent medullary cords. Medullary lymphatic channels adjacent to active cortical areas in the

Downloaded from http://rup.silverchair.com/jem/article-pdf/119/6/955/1081501/955.pdf by guest on 05 February 2023

draining nodes occasionally contained large pyroninophilic mononuclear cells with prominent nucleoli similar to those seen in the cortex. These cells were not seen in the graft or the infiltrate in the graft bed.

In these early examples, there was little evidence of germinal center formation. Later in the course of rejection, and especially in the older animals of the series, including those receiving sterile fresh fetal skin grafts, the formation of germinal centers was more prominent, and intrafollicular pyroninophilia was relatively decreased. In contrast to the reactive lymph nodes of animals receiving antigenic stimuli with accompanying antibody and gamma globulin production, the reactive nodes in the grafted fetuses failed to show production of immature plasma cells.

Protein Response Accompanying Graft Rejection.—It appeared desirable to determine whether the lymphoid hyperplasia that accompanies homograft rejection in the fetal lamb was associated with the production of significant amounts of immunoglobulins. In an earlier paper (6) evidence was presented that the normal fetal lamb forms small amounts of β_{2M}-globulin but no typical 7S γ- or β_{2A}-globulins. The lymphoid response to antigenic and adjuvant injections was invariably accompanied by the formation of 7S γ- or β_{2M}-immunoglobulins, often in appreciable quantities.

Among the fetuses engaged in homograft rejection, no significant increase in circulating β_{2M}-globulin was found above normal levels. In no instance were either β_{2A}- or 7S γ-globulins detected in the sera of these animals. These data were confirmed by the absence of production of significant amounts of these proteins by the reactive lymph nodes grown in tissue culture in the presence of radioactive amino acids (6), and they accord well with the absence of immature and mature plasma cells in the nodes and graft beds.

DISCUSSION

The data presented in this paper confirm and extend the earlier observations of Schinkel and Ferguson (7) that the fetal lamb *in utero* is able to reject allogeneic skin grafts. Orthotopic skin homografts applied to fetal lambs about the 65th day of gestation were accepted and retained for up to 21 days without signs of the intervention of an active rejection process. In contrast, all grafts applied after the 77th day of gestation were accepted initially and then effectively rejected within 7 to 10 days. The rate of rejection and the histologic picture in the graft and its bed and in the reactive draining lymph node were similar to those described for specific homograft rejection in adults of other species.

In an earlier paper of this series (5), it was made clear that the fetal lamb did not suddenly accede to full immunologic competence after an earlier "null" state. Rather, the fetus was observed to respond to antigenic stimuli in a stepwise fashion, related in some unknown manner to the nature of the antigen

Downloaded from http://rup.silverchair.com/jem/article-pdf/119/6/955/1081501/955.pdf by guest on 05 February 2023

involved. Thus, there seems to exist a "hierarchy" of antigens. The response to some, such as bacteriophage ϕX and ferritin, occurs at an early stage of gestation long before the fetus manifests any response to other antigens such as ovalbumin. Antigens such as diphtheria toxoid and *Salmonella typhosa* were not observed to elicit a fetal response at any time during gestation. Antibody formation to these substances did not appear until many weeks after birth. In this respect, ovine histocompatibility antigens appeared to occupy an intermediate position in the antigenic hierarchy. It is thus apparent that no special case can be made in ovine ontogeny between the homograft reaction as one form of immunologic response and antibody formation as another.

The absence of homograft rejection in those animals grafted at about 65 days' gestation raises a problem similar to that posed by the antibody response of the fetal lamb. Does the inability to form antibody to a given antigen or to reject the skin graft prior to some critical time reflect the induction of tolerance to these antigens? Or is it possible that the immature animal is merely immunologically incompetent to respond to the antigen and, in fact, does not "recognize" it in an immunologic sense? Any interpretation of immunologic tolerance, whether involving the destruction of specific clones of cells (12) or the "clogging" of the mechanism of a cell (13), implies a prior competence on the part of the animal involved. There must also be in the developing fetus, however, a period prior to the differentiation of its immunocytologic capabilities when it exists in a true null state, unable to respond in any manner to antigenic stimulus. The retention of a graft in position for 21 days on fetus 30-41 (sacrificed on the 88th day) is certainly significant, for at the same gestation age fetus 30-52 was already rejecting a graft applied 11 days previously. This result could reflect either immunologic tolerance of the graft on the part of this animal or the decreased susceptibility to rejection of a graft that has been well accepted and retained for a period of time (14).

While the fetal lamb is able to form antibody in response to certain antigens, as has been shown earlier (5), there is little question that in most instances this initial intrauterine response is somewhat less efficient than that of the adult. It is therefore surprising that once the fetus develops the ability to reject homografts, it does so with the rapidity and apparent competence of the adult, furnishing the full range of cellular events, graft invasion, and breakdown within 7 to 10 days of application. In this respect, the acquisition of competence by the fetus appears to be complete despite its relatively abrupt appearance. This was true regardless of whether the graft came from an unrelated fetus or adult, or from its own mother.

A final point may be raised relative to the mechanism of homograft rejection and its possible dependence on circulating antibody (15). In other species, appearance of plasma cells in the reactive draining lymph node and in the graft bed itself has been cited in support of the notion that circulating antibody

Downloaded from http://rup.silverchair.com/jem/article-pdf/119/6/955/1081501/955.pdf by guest on 05 February 2023

is produced during and ultimately participates in the rejection process. The fetal lamb offers a unique opportunity to study this question, since the ovine fetus is to all intents and purposes an immunologic virgin with extremely immature lymphoid tissue and little or no immunoglobulins in its circulation.

It is therefore of special significance that when care was taken to transplant sterile skin from a fetal donor, no plasma cells could be found in either the draining nodes or the graft bed at any time prior to, during, or after rejection. Neither could any increase in circulating immunoglobulins be observed in these fetuses, a fact confirmed by tissue culture experiments performed on various organs. The suggestion that the homograft reaction is a cellular form of response is further borne out by the preliminary results reported elsewhere (8) indicating that the fetal lamb is able to reject a homograft in a normal fashion despite the presence of excess rabbit anti-sheep β_{2M}- and 7S γ-globulins in its circulation at the time of rejection. All of these data point to the possibility that conventional circulating antibodies do not play an *obligatory* role in the rejection of solid tissue homografts.

SUMMARY

The fetal lamb was found to reject orthotopic skin homografts applied at any time after the 77th day of gestation. Prior to this, grafts remained in place without stimulating any detectable immunologic response. Once the fetus achieves the ability to reject the graft, the process occurs with the same competence and rapidity as in the adult. Graft rejection in the fetal lamb is unaccompanied by formation of plasma cells or by the production of typical immunoglobulins, thus supporting the suggestion that circulating antibody does not play an obligatory role in the process.

BIBLIOGRAPHY

1. Uhr, J. W., Development of delayed hypersensitivity in guinea pig embryos, *Nature*, 1960, **187**, 957.
2. La Via, M. F., Rowland, D. T., and Block, M., Antibody formation in embryos, *Science*, 1963, **140**, 1219.
3. Silverstein, A. M., and Lukes, R. J., Fetal response to antigenic stimulus. I. Plasmacellular and lymphoid reactions in the human fetus to intrauterine infection, *Lab. Inv.*, 1962, **11**, 918.
4. Eichwald, H. F., and Shinefield, H. R., Antibody production by the human fetus, *J. Pediat.*, 1963, **63**, 870.
5. Silverstein, A. M., Uhr, J. W., Kraner, K. L., and Lukes, R. J., Fetal response to antigenic stimulus. II. Antibody production by the fetal lamb, *J. Exp. Med.*, 1963, **117**, 799.
6. Silverstein, A. M., Thorbecke, G. J., Kraner, K. L., and Lukes, R. J., Fetal response to antigenic stimulus. III. Gamma globulin production in normal and stimulated fetal lambs, *J. Immunol.*, 1963, **91**, 384.

Downloaded from http://rup.silverchair.com/jem/article-pdf/119/6/955/1081501/955.pdf by guest on 05 February 2023

7. Schinkel, P. G., and Ferguson, K. A., Skin transplantation in the fetal lamb, *Australian J. Exp. Biol. and Med. Sc.*, 1953, **6,** 533.
8. Silverstein, A. M., Prendergast, R. A., and Kraner, K. L., Homograft rejection in the fetal lamb: The role of circulating antibody, *Science*, 1963, **142,** 1172.
9. Brambell, F. W. R., Passive immunity in young mammals, *Biol. Rev.*, 1958, **33,** 488.
10. Billingham, R. E., The storage of skin, *in* Preservation and Transplantation of Normal Tissues (G. E. W. Wolstenholme and M. P. Cameron, editors), London, J. and A. Churchill Ltd., 1954.
11. Medawar, P. B., The behaviour and fate of skin autografts and skin homografts in rabbits, *J. Anat.*, 1944, **78,** 176.
12. Burnet, F. M., The Clonal Selection Theory of Immunity, Nashville, Vanderbilt University Press, 1959.
13. Szilard, L., The molecular basis of antibody formation, *Proc. Nat. Acad. Sc.*, 1960, **46,** 293.
14. Woodruff, M. F. A., *in* Biological Problems of Grafting (F. Albert and P. B. Medawar, editors), Oxford, Blackwell Scientific Publications, 1959.
15. Converse, J. M., and Rogers, B. O., Fifth tissue homotransplantation conference, *Ann. New York Acad. Sc.*, 1962, **99,** 335.

Downloaded from http://rup.silverchair.com/jem/article-pdf/119/6/955/1081501/955.pdf by guest on 05 February 2023