that anterior pituitary viability for this group was significantly less than that for the 2.5 ml group. The lower viability rating of the 2.0 ml group was believed to be due to the culturing of larger pituitaries in some of the experiments. These larger pituitaries when examined histologically had an area of central necrosis that could be attributed to the lack of nutrients and/or gas environment in this area. It was necessary, therefore, that pituitaries be adequately fragmentized to allow for proper nutritional and gaseous exchange to all cells.

Addition of insulin to the medium in some of the experiments in the bicarbonate study appeared to have no effect on lactogen production. This observation was in accord with reports of other workers(10).

In the temperature study lactogen production increased with increasing temperature to 39°C, when it decreased below that noted for 37°C. This decrease was believed to be due to tissue damage, since AP viability ratings were significantly lower.

Summary. The influence of level of sodium bicarbonate and temperature on lactogen production by rat anterior pituitaries cultured *in vitro* was studied. Pituitaries were cultured for 3 days in the chemically defined mixture 199. When compared with the 2.5 ml group, addition of 2.0 ml of 2.8% $NaHCO_3$ per 25 ml medium resulted in a 19.2% higher lactogen production. Anterior pituitaries cultured at 37°C produced 22.5% more lactogen than those cultured at 35°C, and 16.7% more than those cultured at 39°C. At the highest temperature, AP tissue damage was noted. When the bicarbonate level and culture temperature were optimum for lactogen production, anterior pituitaries synthesized and released 11.2 times more lactogen than that which was initially introduced into the culture system with the pituitary gland.

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Possible Role of Leucocyte Granules in the Shwartzman and Arthus Reactions.* (28879)

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Polymorphonuclear leucocytes appear to play an essential role in the pathogenesis of the Shwartzman and Arthus reactions. Both are characterized by extensive infiltration of the involved tissues by these cells prior to the development of hemorrhage and necrosis, and both can be prevented by agents causing polymorphonuclear leucopenia(1-3). In the Arthus reaction, Cochrane and Weigle(4) have shown that temporary leucopenia does not prevent the formation of antigen-antibody complexes in the skin, but tissue destruction begins to occur only when leucocytes appear in the area.

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Cohn and Hirsch(5) demonstrated that the granules of leucocytes possess the properties and enzymatic composition of lysosomes. De Duve(6) and others have suggested that the acid hydrolytic enzymes contained in lysosomes may be of importance in production of damage to cells or tissues, if released into the cell sap or extracellular space. Daems and Oort(7), using electron microscopy, have shown that degranulation of leucocytes takes place during the development of the Arthus reaction. In the case of the Shwartzman reaction, Weissmann and Thomas(8) found that endotoxin has the property of causing instability of lysosomes and release of lysosomal enzymes *in vivo*.

In the present study, it has been found that intradermal injection of granules isolated from rabbit polymorphonuclear leucocytes prepares the skin for a reaction resembling the Shwartzman reaction, and also causes marked enhancement of the Arthus reaction.

Materials and methods. Rabbit leucocyte granules were prepared by the method of Cohn and Hirsch(5). Peritoneal exudates were obtained by intraperitoneal injection of 300-400 ml of 0.1% glycogen, or by injection of larger volumes of sterile, pyrogen-free, physiological saline. The leucocytes, usually 90-95% granulocytes, were collected by centrifugation, washed once in cold saline, then in 0.34 M sucrose, and finally suspended in iced sucrose and homogenized lightly with a syringe. When opalescence and viscosity appeared, indicating rupture of the cells and release of granules, the cell debris was removed by light centrifugation and the granule fraction collected by centrifugation at 12,000 $\times$ *g* for 30 minutes. Bacteriological cultures of the peritoneal fluid and the final granule suspensions were negative in all instances.

For injection, the washed granules were resuspended in 0.34 M sucrose to a volume representing a 100-fold concentration of the original peritoneal exudate. Thus, for most preparations used, each ml of granule suspension represented the yield from approximately 5×10^7 leucocytes.

The injections were given intradermally, in amounts of 0.5 ml, into the clipped abdominal skin of albino hybrid rabbits, averaging 1.5 kg in weight.

For the Shwartzman reaction, a preparation of *E. coli* lipopolysaccharide (Difco) was injected by vein at various times before and after preparation of the skin with granules. The dose was 100 μg/kg.

For the Arthus reaction, bovine serum albumin (BSA) was injected by vein (25 mg/kg), and 30 minutes later rabbit anti-BSA serum (0.1 ml) was injected intradermally at skin sites previously prepared with granules, and at control sites.

Polymorphonuclear leucopenia was produced by intravenous injection of nitrogen mustard (HN_2), in a dose of 1.75 mg/kg. White cell and differential counts were made each day to determine the extent of leucopenia.

Results. Shwartzman reaction. When endotoxin was injected between 4 and 12 hours after preparation of the skin with granules, extensive lesions of hemorrhagic necrosis occurred within 3 hours in approximately 50% of 84 animals tested (Table I). These lesions, in their gross appearance, were indistinguishable from the usual Shwartzman reaction produced by preparing skin with endotoxin. In 26 rabbits, less conspicuous lesions appeared, consisting of scattered small petechiae in circular areas measuring 2-3 cm in diameter. When such areas were lightly pressed with a cotton swab, the petechiae immediately increased in number and often coalesced to form confluent hemorrhages, indicating an increased fragility of small vessels throughout the prepared area.

TABLE I. Production of Skin Hemorrhage by Leucocyte Granules.

Lesions	Time interval* 0-4 hr	4-12 hr	12-24 hr
Hemorrhagic necrosis	0/12†	43/84	2/12
Fragility‡	0/12	26/84	2/12

* Refers to interval between intradermal injection of granules and intravenous injection of endotoxin.

† Numerator refers to number with lesions, denominator to number in group.

‡ Fragility indicated by the appearance of petechiae after light pressure over prepared area.

Histological studies of the lesions showed diffuse hemorrhage, involving all layers of the dermis. Many of the venules and capillaries were greatly dilated, and some contained thrombi of leucocytes and platelets similar to those described by Stetson(9) in the classical Shwartzman reaction. However, there was little or no evidence of infiltration of the prepared area by polymorphonuclear leucocytes. Giemsa-stained sections revealed clusters of red granules in some areas, which had an appearance consistent with that of leucocyte granules.

Control skin areas which were prepared with granules for the same length of time, but without intravenous endotoxin, showed no hemorrhages, and little or no gross evidence of a cellular reaction to the granules. Giemsa-stained sections showed many more masses of intact granules than were seen in the hemorrhagic lesions provoked by endotoxin. The significance of this finding is in doubt, since the extensive extravasations of blood into the latter tissues made identification of granules more difficult.

It was found that the debris of leucocytes resulting from rupture in 0.34 M sucrose could be rendered almost free of granules by repeated washing in cold sucrose, with centrifugation at 800 *g* for 10 minutes between each wash. The activity of a suspension of relatively isolated granules could therefore be compared with that of the remaining cell solids free, or almost free, of granules. Accordingly, 8 rabbits were prepared with the usual granule suspension on one side of the abdomen, and with a suspension of the total cellular material after 4 washings in sucrose, on the other. Endotoxin was injected 6 hours after preparation. Fig. 1 illustrates the results obtained in all rabbits; the sites prepared with granules showed hemorrhagic necrosis, while those prepared with granule-free nuclear and cytoplasmic debris from the same cells showed no reaction, or very small areas of petechial hemorrhage.

It has been shown that an injection of adrenalin into skin areas previously prepared with endotoxin will produce hemorrhagic necrosis(10,11). Similar reactions occurred when adrenalin (0.1 ml of 1-1000 solution) was injected into the center of areas prepared 4 hours earlier with leucocyte granules. Histologically, these lesions showed diffuse hemorrhage without leucocytic infiltration or thrombosis of venules or capillaries.

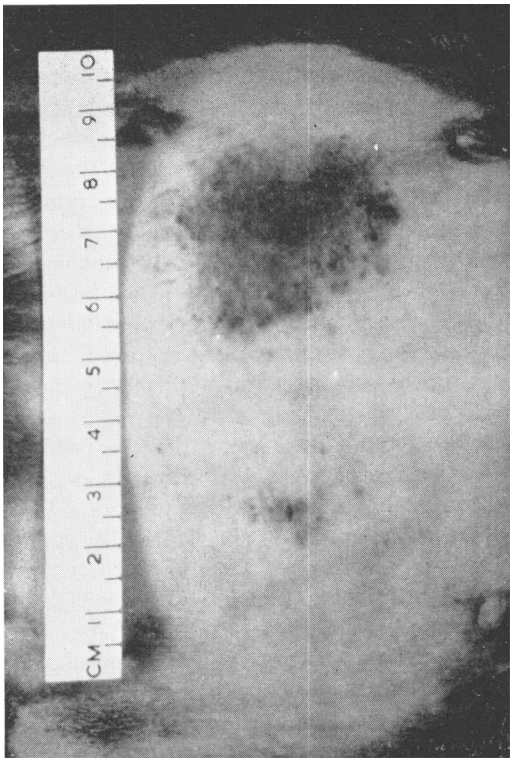

FIG. 1. (Top) Lesion produced by preparation of skin with leucocyte granules followed 6 hours later by intravenous endotoxin. (Bottom) Small petechiae at skin site prepared with leucocyte debris after repeated washing in 0.34 sucrose. Centimeter rule indicates size of lesions.

The time interval between injections of granules and endotoxin was of much importance in determining the outcome. When it was less than 4 hours, or longer than 15 hours, no hemorrhagic reaction could be elicited by endotoxin. Similarly, the optimal period for the hemorrhagic lesions produced by adrenalin was 4-12 hours after granules.

Polymorphonuclear leucopenia, induced by HN_2, appeared to prevent the lesions caused by granules and intravenous endotoxin. In a group of 12 rabbits, with granulocyte counts of 800 per cu mm or less on the day of testing, no hemorrhages or evidence of increased fragility of blood vessels occurred. This observation suggests that leucocytes or platelets

in the circulating blood may also be necessary for these reactions, perhaps, as suggested by Stetson(9), in production of local ischemia by thrombosis.

The systemic effects of leucocyte granules were examined in order to explore the possibility of contamination of the granule preparations with endotoxin, perhaps derived from peritoneal exudate. Intravenous injection of 5 ml of granule suspension failed to produce fever, leucopenia, or the reaction of adrenalin-necrosis previously reported(11) to be a fairly sensitive indicator for the presence of endotoxin. Moreover, intravenous granules did not provoke either the local or generalized Shwartzman reaction in rabbits appropriately prepared with endotoxin. It is unlikely that an endotoxin sufficiently potent to cause the skin reactions under study could be present in the granule suspensions without producing any of these systemic effects.

The Arthus reaction. When reversed passive Arthus reaction was elicited at skin sites prepared 6 hours earlier with granules, the size and intensity of the reaction were uniformly greater than those at control sites in the same animals. With the dose of BSA and antibody employed, the reactions at control sites in 12 rabbits consisted of mild erythema measuring 1-1.5 cm in diameter. In contrast, the lesions in sites prepared with granules were grossly hemorrhagic in 6 of the animals and deeply erythematous in the others, and measured 2-4 cm in diameter. In both areas the lesions began to appear at about 6 hours after injection of antibody, and were fading by the end of 24 hours.

No reactions occurred in control rabbits in which anti-BSA rabbit serum was injected into granule-prepared skin without antigen being given by vein. Moreover, rabbits receiving BSA by vein and anti-ovalbumin in the skin showed no reactions.

Polymorphonuclear leucopenia was induced by HN_2 in 12 rabbits, and tests for the reversed passive Arthus reaction in granule-prepared and normal skin sites were performed as above. The reactions were completely negative at the control sites in all animals, while 8 of the 12 showed mild but definite reactions in skin areas prepared with granules. In 3 of these, the reactions were hemorrhagic. Although these Arthus reactions were uniformly less intense than those seen in animals with normal leucocyte counts, it seemed clear that the preparation of skin with granules permitted elicitation of reactions in spite of leucopenia.

Effect of hydrocortisone. The capacity of hydrocortisone to increase the stability of lysosomes and prevent their rupture by various agents has been demonstrated in several experimental models(8,12). The effect of this agent on the reaction caused by granules and endotoxin was studied. Sixteen rabbits were prepared with intradermal granules and given intravenous endotoxin 6 hours later. Eight of the animals were injected by vein with 10 mg hydrocortisone immediately before granules, and again immediately before endotoxin. No lesions occurred in any of the treated animals, while 7 of the 8 untreated controls developed hemorrhagic necrosis.

Inactivation of granules. Heating suspensions of granules at 80°C for 30 minutes caused disappearance of all activity when tested for the Shwartzman reaction. Freezing and thawing 10 times in a dry ice-alcohol bath also inactivated the preparations, presumably by rupturing the granules.

Reactions with autologous granules. Granule fractions were prepared from the peritoneal exudates of 3 rabbits, and used for preparation of the skin in each individual donor. Intravenous endotoxin caused hemorrhagic lesions which were similar in all respects to the lesions produced by granules from other animals.

Discussion. It would appear that the isolated granules of polymorphonuclear leucocytes can be substituted for endotoxin in the local Shwartzman reaction, and can play the role of intact leucocytes in the Arthus reaction. However, there is always danger in assigning the functions of endotoxin to extracts or other products of animal tissues, in view of the ubiquity of gram-negative bacteria and their products; this is particularly the case for exudates produced by peritoneal irritation, where the bacterial flora of the intestinal tract is such a short distance away. Although such contamination cannot be entirely

excluded in the present study, the following points can be made against the likelihood of this: 1) cultures of the exudates and granule suspensions were negative; 2) the activity of granules in the Shwartzman reaction was destroyed by heating at 80°C for 30 minutes, in contrast to the uniform thermostability of endotoxin; 3) inflammatory infiltration of the skin by granulocytes was not produced by the granules, while intradermal endotoxin brings about vigorous inflammation; 4) intravenous injection of granule suspensions did not produce fever, leucopenia, or the reaction of adrenalin-necrosis(11), all of which are reasonably sensitive indicators for the presence of endotoxin; and 5) activity was demonstrable only in the granule fraction of the leucocytes, while the cell debris remaining after repeated sucrose washings, which comprised a considerably larger mass of tissue than the granule fraction, was inactive.

The necessity for a time interval of 4 hours or longer between injections of granules and endotoxin is unexplained. This interval is considerably shorter than that involved in the conventional Shwartzman reaction (18-30 hours), but is nevertheless long enough to raise a question as to the primary nature of the action of granules in the reactions under study. It may be that time is required for the injected granules to be transported within the skin tissue to special targets, conceivably to the environs of small blood vessels. It is not known whether uptake of the granules by tissue cells takes place *in vivo*. In experiments with rabbit peritoneal macrophages in tissue culture, we have observed rapid phagocytosis of polymorphonuclear leucocyte granules on several occasions; the ingested granules seemed to remain intact and could still be visualized as late as 18 hours after being ingested.

The prevention of the granule-induced Shwartzman reaction by hydrocortisone may be due to the known capacity of the hormone to cause increased stability of lysosomes(8,12), although it is also possible that hydrocortisone may be interfering with some other, systemic action of endotoxin. The protective action of nitrogen mustard against the Shwartzman reaction indicates that circulating leucocytes, or perhaps platelets, are necessary for this reaction. In the case of the passive Arthus reaction, preparation of the skin with granules produced hemorrhagic reactions in the absence of circulating leucocytes, suggesting that the events leading to hemorrhage are mediated by the local interaction between granulocytes and antigen-antibody complex, as proposed by Stetson and Good(2), Cochrane and Weigle(4), and Daems and Oort(7).

The finding that repeated freeze-thawing inactivated the granule preparations for the Shwartzman reaction indicates that the activity is not simply due to the presence of an excess of lysosomal enzymes in the skin. It is more likely that the granules have their function as vehicles for highly concentrated enzymes, perhaps cathepsins, brought into close proximity with vulnerable tissue structures. It is suggested that rupture of the granules may result from local ischemia in the Shwartzman reaction, and also in the reactions of hemorrhagic necrosis induced by adrenalin. In the Arthus reaction, it may be that contact of granules with antigen-antibody precipitates leads to rupture and subsequent damage to tissue elements in the vicinity; the electron microscopic studies of Daems and Oort suggest this possibility.

Although it is impossible to be certain that it is the leucocyte granules themselves that are responsible for the reactions of hemorrhagic necrosis, some support for the view is provided by the finding that the cellular debris rendered free of granules by repeated washings in sucrose, although comprising a mass of cell solids much heavier than the granule suspensions, were incapable of preparing skin for the Shwartzman reaction.

The observation that autologous granules are active in preparation for the Shwartzman reaction should exclude the possibility that the reactions are due to intrinsic hypersensitivity of individual rabbits for the leucocyte products of others.

Conclusion. Preparation of the skin of rabbits by intradermal injection of the granule fraction obtained from peritoneal granulocytes, followed by intravenous injection of endotoxin, results in lesions of hemorrhagic

necrosis which resemble the local Shwartzman phenomenon. An injection of granules into the skin enhances the reactivity of this area to the reversed passive Arthus phenomenon, and makes it possible to elicit this reaction in leucopenic animals. It is suggested that one or more of the acid hydrolases contained in granules may be implicated in the pathogenesis of vascular damage in the Shwartzman and Arthus reactions.

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Respiratory Syncytial Virus: Observations on Antigenic Heterogeneity.* (28880)

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During the spring of 1962, we recovered 24 strains of RS virus from children admitted to hospital during a respiratory illness (1). Several other strains were recovered later in the same year from children in an orphanage. These strains were so poorly neutralized by prototype (Long) antiserum that they would have been missed had this criterion been the sole means of identification.

All RS strains until recently appeared to be close antigenic relatives. Strains recovered in 1962 suggested that antigenic variants serologically distinguishable from the prototype may be encountered during outbreaks involving human beings. In contrast with the 1962 strains, another isolated in 1963 was serologically closely related to "Long." Recently, Coates *et al*(2) reported on antigenic dissimilarities of 2 RS viruses. We are adding another example of antigenic heterogeneity among these viruses.

Materials and methods. Virus stocks. Strains entered in the study are described in another paper(3).

Immune sera. For the prototype strain "Long." Two ml of undiluted virus stock was inoculated intraperitoneally into each of 3 rabbits, 6 times at intervals of 7 days. Animals were bled on the 49th day. A second series of immunizations identical with the first was started 4 to 5 weeks after the first bleeding. Animals were bled 7 weeks afterwards. After the 2nd bleeding 3 "boosters" were given each a week apart. Rabbits were exsanguinated 5½ months after onset of immunization program. *For strain "87."* This virus, our 1962 prototype, was recovered in Kansas City from an 18-month-old infant with laryngotracheobronchitis. Antiserum was

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