Brit. J. Pharmacol. (1957), **12**, 427.

A COMPARISON OF THE LOCAL EFFECTS OF VARIOUS INTRAMUSCULAR INJECTIONS IN THE RAT

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(RECEIVED APRIL 26, 1957)

A method is described for the assessment of the local reaction produced by intramuscular injections. This method involves the histological examinations of the injection site after injection of material into the hamstring muscles of the rat. The results obtained with both aqueous and oily materials are described; the correlation of these results with clinical observations in man is discussed.

The phenomenon of muscle regeneration has attracted much study and experimental work in the past. The literature on this subject has been fully reviewed by Adams, Denny-Brown and Pearson (1953). The most recent study is that of Walton and Adams (1956), who were concerned with the differences in regenerative capacity of normal, denervated and dystrophic muscle after a standard injury. In this type of study investigators have naturally been concerned to inflict an injury as standard as possible, whether by crushing, incision, or the injection of highly injurious material such as alcoholic solutions. It is surprising, in view of the frequency with which drugs are administered by intramuscular injection, that few studies have been published on the amount and type of injury produced by substances likely to be given by intramuscular injection therapeutically. Hawking (1943, 1944, 1945) compared the effects of various salts of quinine and mepacrine on rabbit muscle, but included no standard materials as a basis of reference, while Lüthy (1955) and von Hochsteffer (1955) both quote the unpublished results of another investigator (Shallock) as the authority for various changes after the intramuscular injection of various pharmaceuticals. Beresford, Golberg, and Smith (1957) published a careful investigation of local tissues after the injection of iron formulations, but they too did not study the effects of any other commonly used injection to provide a standard of reference. A similar criticism applies to the less detailed studies of Glückert and Benoit (1952) on the effects of sulphonamide injections, while Brown, Wilder, and Schwarz (1944), although using several types of injection, were solely concerned with the effects of oily injections. Nevertheless, clinically it is evident that marked differences occur between the reactions produced locally by various agents and indeed between various formulations of the same agent. Some injections rarely give rise to pain or swelling at the injection site, while other substances produce such severe local changes as to preclude their general use in this manner. In the case of a new drug or of a new formulation of a drug intended for intramuscular injection, this aspect is important and some means of studying such local reactions must be developed. We here describe the method used in these laboratories and the results obtained with some simple solutions and with some substances commonly administered by intramuscular injection. This method was developed here some years ago by Miss J. M. Gates and Dr. E. Weston Hurst. Although it does not provide ideal material for fundamental studies on muscle regeneration, for which it is not designed, it does permit the careful comparison of the irritancy of various materials under conditions simulating those of human use.

MATERIALS AND METHODS

Preparation of Solutions

Stock Carbon Black Suspension.—A stock suspension of carbon black (Darco G.60) 1 in 50 in water for injection *B.P.* was made and sterilized by autoclaving at 10 lb. pressure for 30 min. It is essential to use only the purest available carbon black. Several examples tested by us gave severe local reactions. Darco G.60 was satisfactory in this respect.

Saline 0.9% w/v.—Saline solution (0.9% w/v sodium chloride) in water for injection *B.P.* con-

taining carbon black 1 in 600 (such that 3 ml. of final solution contained 2.75 cc. of saline solution and 0.25 cc. of stock carbon black suspension 1 in 50) was prepared and sterilized by autoclaving at 10 lb. pressure for 30 min.

Test Solution.—The solution to be tested containing carbon black 1 in 600 was prepared in the same manner as the saline solution, and sterilized by autoclaving at 10 lb. pressure for 30 min. If the material was thermo-labile, the sterile carbon black suspension 1 in 50 was added aseptically to the sterile test solution. The tests described in this paper were made either on aqueous solutions prepared in the laboratory or on material produced for sale by Imperial Chemical Industries Limited, Pharmaceutical Division, except for the oils, which were derived from various sources.

Methods of Injection and Dissection

A minimum of 5 rats is required for each test. The rats should be from the same litter and each weigh approximately 100 g. Since a few of the injections may not be fully intramuscular, but in fascial planes and subcutaneous tissue, it is our practice to use 10 rats for each test.

The hair was clipped from the skin of the thighs of the hind legs of the rats. The rat was then held as for intraperitoneal injection and the injection sites were swabbed with antiseptic solution. The posterior thigh muscle of the left leg was then injected with 0.2 cc. of sterile saline containing carbon black (1 in 600) using a sterile syringe and needle. Similarly, the muscle of the right leg was injected with 0.2 cc. of sterile test solution containing carbon black (1 in 600) using another sterile syringe fitted with the same size of needle as that used for the saline injection.

At 2, 6, 24, 48, and 72 hr. after injection, two rats were killed by means of chloroform.

For dissection, the rat was pinned out on the dissecting board in the supine position and the hind legs were again swabbed with antiseptic solution. (It has been found that swabbing facilitates dissection.) The legs were compared externally and any differences noted.

The skin was then reflected from the muscles. Again the legs were compared and any differences noted (such as inflammation, oedema, etc.).

The tendon at the knee joint was gripped with forceps and cut at a point below the forceps. The muscle was removed from the leg by cutting up between the bone and muscle and finally across the muscle above the site of injection. Transverse sections of muscle were then cut, using a new scalpel blade, until the injection site showing carbon black was located. (It was important that the sections be cut using a saw-like movement with the blade, as direct pressure of the blade on the muscle caused damage.) A suitable section showing carbon black in the muscle was then placed between two pieces of filter paper (Whatman No. 50) and immersed in Zenker-acetic acid fixative. If the injection was truly intramuscular, the carbon black appeared dull in the muscle. If the carbon black appeared shiny in the cut surface of the muscle, it was probable that the injected material was lying between the muscles, and the tissue was not suitable for histological examination. If this was the case another rat was sacrificed.

It was essential that all injection material should be sterile and given aseptically. It was also essential that great care should be taken not to damage the muscle during dissection.

The fixed muscles were processed through paraffin; 5 μ sections were cut and stained with Anderson's haematoxylin and eosin.

Results

Normal Saline, Water, and Hypertonic Saline.—As might be predicted, sterile normal saline gave rise to the least amount of local disturbance seen in this test. Two hours after the intramuscular injection of normal saline with the marker carbon there was separation of muscle bundles around the injection site; a few wisps of eosinophilic material between these muscle bundles indicated the presence during life of some oedema fluid. Along the needle track and in contact with the injected material a few muscle fibres were seen to be disrupted, and their cytoplasm to be more eosinophilic than normal. In a cross-section of such an injection site no more than 6 to 10 fibres were seen to be so affected. Even 2 hr. after the injection, a few polymorphs can be seen particularly in the region of the carbon granules, but also in the area of oedema (Fig. 1*a*). Six hours after the injection the evidence of oedema was diminished, and polymorphs were more frequent. By 24 hr. the oedema had subsided and polymorphs were present in moderate numbers around the damaged fibres and the carbon pigment. At this time active phagocytosis by mononuclear cells of the carbon and of damaged muscle fibres could be seen, and very occasional fibroblasts were also present near the injection site. At 48 hr., phagocytes had replaced polymorphs as the predominant cell in the reaction, and the majority of the necrotic muscle had been removed by their activities. By this time eosinophils were seen in moderate numbers. Fibroblastic proliferation was brisk, and the injected carbon was beginning to be surrounded by a fibrous capsule. At 72 hr. this capsule was complete; it involved also a few muscle bundles in the immediate vicinity of the carbon. The majority of the phagocytes had disappeared, the whole injection site now appeared tidy, and the reaction had subsided completely (Fig. 1*b*).

Sterile distilled water gave rise to a surprisingly severe reaction when injected intramuscularly.

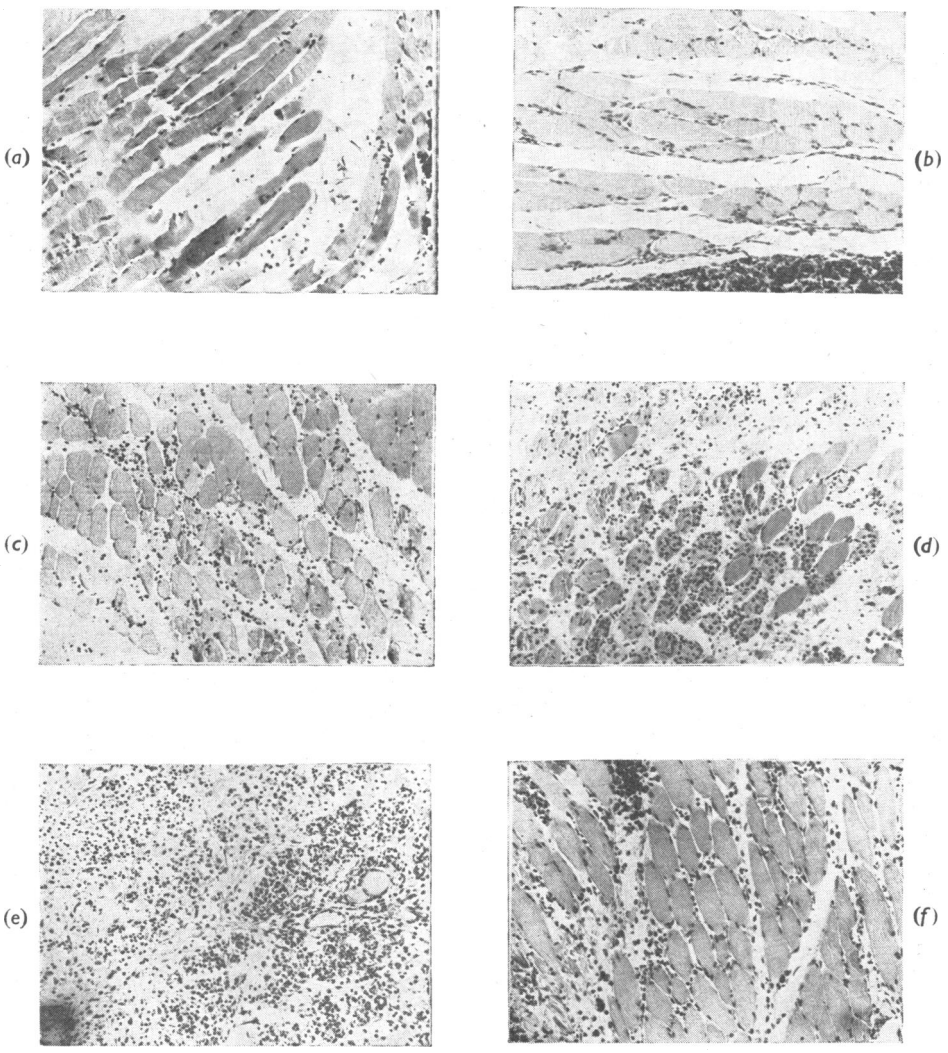

FIG. 1.—Photomicrographs of muscle fibres stained with haematoxylin and eosin from the hamstring muscles of rats receiving intramuscular injections. Magnification in all photomicrographs, × 75. The time shown in parentheses indicates the period between injection and removal of specimen for histological examination. (*a*), Isotonic saline (2 hr.). Slight oedema and polymorphonuclear infiltration close to carbon. (*b*), Isotonic saline (72 hr.). A thin fibrous capsule surrounds the carbon. (*c*), Distilled water (6 hr.). Considerable oedema and polymorphonuclear infiltration. Note the invasion of occasional necrotic muscle fibres by polymorphonuclear leucocytes. (*d*), Distilled water (24 hr.). Extensive invasion of necrotic muscle fibres by polymorphonuclear leucocytes and phagocytes. (*e*), Distilled water (72 hr.). Considerable polymorphonuclear and phagocyte activity. No damaged muscle remains unattacked. (*f*), Hypertonic saline (72 hr.). Rather extensive fine fibrosis. Compare with (*b*).

Two hours after the injection, the carbon was surrounded by a zone some 2 mm. in diameter in which the muscle bundles, and the individual fibres within the bundles, were separated by oedema. In this zone numerous fibres appeared disrupted and many other fibres showed altered staining properties, some being swollen and eosinophilic, others markedly basophilic. At the edge of this markedly oedematous zone the muscle bundles were separated by oedema, but the bundles themselves were not disrupted. At this time polymorphs were present in quite large numbers throughout the oedematous area ; others could be seen in the process of migration from vessels in the area.

Six hours after the injection the oedema had subsided somewhat, although much still remained. By this time numbers of muscle fibres throughout the affected area were swollen and obviously necrotic; in some, invasion of the cytoplasm by polymorphs had commenced (Fig. 1*c*). Occasional macrophages could now be seen. By 24 hr. the acute inflammatory reaction was fully established and the whole area around the injection site was infiltrated with polymorphs and macrophages. This infiltration was most intense around the carbon, but was marked throughout the oedematous area. Polymorph invasion and phagocytosis of damaged muscle fibres was well advanced (Fig. 1*d*). At 48 hr., polymorph infiltration was still prominent, but phagocytes had increased in number and fibroblasts were now appearing, particularly around the carbon, but also around damaged muscle fibres. Occasional basophilic muscle fibres with central nuclei were now seen. These were much more numerous at 72 hr., when large areas of such proliferating muscle were seen. At this time, fibroblastic proliferation was marked, but evidence of a continuing acute inflammatory response, with polymorph and phagocyte infiltration of the damaged area, could still be seen. Damaged and necrotic muscle fibres were still present although in small numbers, and active phagocytosis of these was proceeding (Fig. 1*e*). Sections from animals in which the injection had not been fully intramuscular demonstrated that a similar intense reaction occurred in the subcutaneous adipose and connective tissue.

By contrast, hypertonic saline (1.7%) gave rise to little more damage to muscle than did isotonic saline. However, the oedema which arose was much more extensive and separated muscle bundles quite remote from the injection site, although the individual fibres in these bundles were not damaged. Inflammatory reaction appeared early, and extended throughout the oedematous area; this area was also involved in the ultimate fibrosis (Fig. 1*f*).

Calcium Gluconate.—Calcium gluconate 10% gave rise to little more tissue damage at the injection site than did saline. About the same number of fibres were necrotic: rather more showed swelling and other signs of damage short of actual necrosis. The cellular response was greater in amount at all times after the injection of calcium gluconate than after the injection of saline; 2 hr. after the injection quite large numbers of polymorphs were seen at the injection site.

Potassium Penicillin.—The injection of potassium penicillin 500,000 units/ml. gave rise to a zone of oedema 3 to 4 mm. diameter at 2 hr. In this zone all the muscle fibres appeared swollen, their cytoplasm was homogeneous and eosinophilic and the nuclei pyknotic. No polymorphs or other cellular reaction could be seen 2 or 6 hr. after the injection (Fig. 2*a*). By 24 hr. small numbers of polymorphs could be seen at the periphery of the damaged area and some also around the carbon at the centre. There was, however, no general invasion of the oedematous area, or of the necrotic muscle, by polymorphs or histiocytes. At 72 hr. this area was still devoid of polymorphs or other cells, although at this time the polymorph reaction around the central carbon was brisk, and at the periphery granulation tissue formation and muscle proliferation were proceeding as well. In some sections at all periods after the injection some damage to blood vessels could be seen, although this was not usually of severe degree.

The injection of potassium penicillin at a strength of 100,000 units/ml., at which strength the solution is isotonic, gave rise to an area of damaged muscle scarcely less than that caused by the stronger solution, nor did the character of the reaction differ appreciably.

Quinine Dihydrochloride.—Two hours after the injection of quinine dihydrochloride the damaged area was 6 to 8 mm. in diameter. Oedema was most marked at the periphery of this zone; at its centre the muscle fibres appeared to have been fixed before separation by oedema fluid could occur. In many sections some haemorrhage could be seen in the central parts of the lesion, and the red cells in some venules appeared damaged, while other veins showed damaged walls. After the injection of quinine dihydrochloride a large proportion of the damaged muscle fibres was strongly basophilic. Cellular reaction at the injection site was first seen 24 hr. after the injection, when it was confined to a sparse polymorph infiltration at the extreme periphery of the damaged area. Even 72 hr. after the injection, there was no inflammatory infiltration of the main bulk of damaged muscle, nor was any reaction to be seen surrounding the carbon (Fig. 2*b*). At this time there was a brisk inflammatory infiltration at the periphery of the damaged area, with muscle proliferation, granulation tissue formation and commencing fibrosis (Fig. 2*c*).

Oily Injections

The agents described above were all aqueous solutions. Non-aqueous injections, as might be expected, produced a different type of reaction at the site of injection. Two examples of this type of reaction are described below.

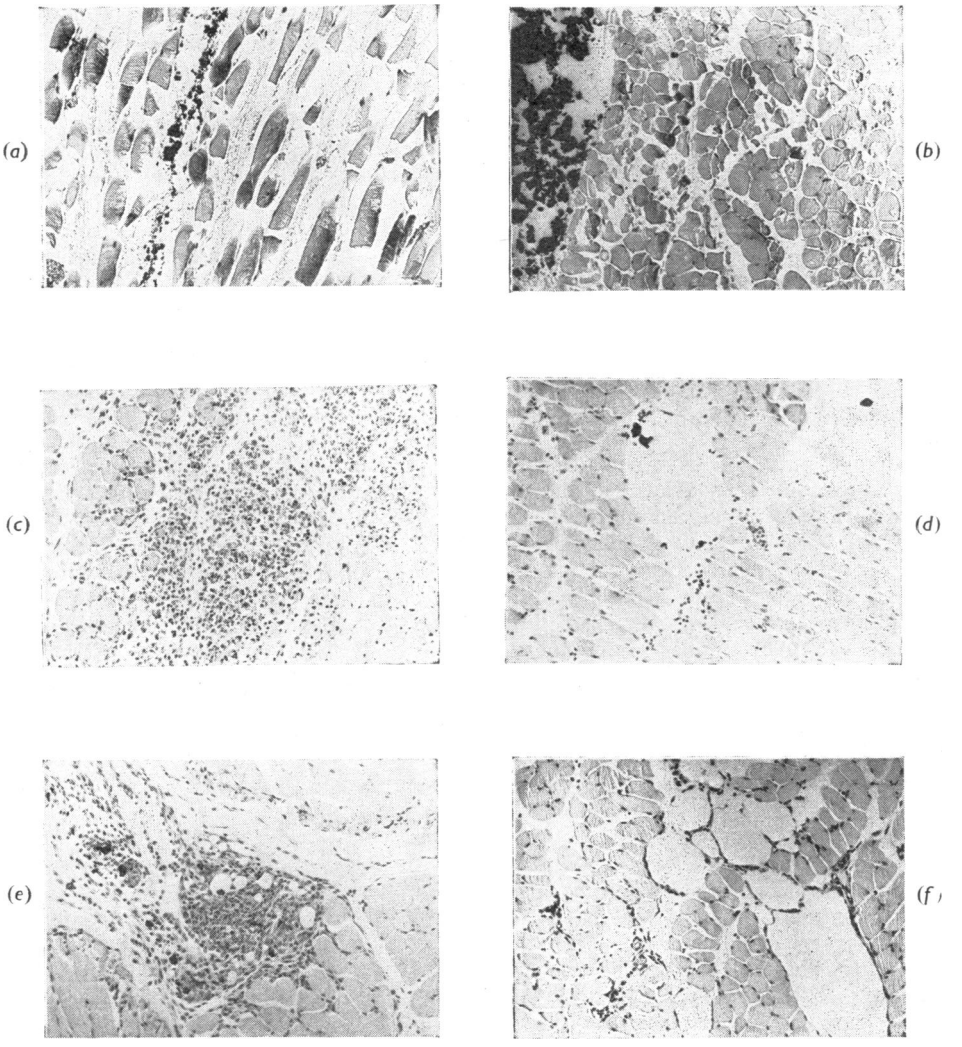

FIG. 2.—Photomicrographs as in Fig. 1. (*a*), Potassium penicillin (6 hr.). Severe muscle damage, no cellular reaction. (*b*), Quinine dihydrochloride (72 hr.). Central portion of lesion showing necrotic muscle devoid of cellular reaction. (*c*), Quinine dihydrochloride (72 hr.). Edge of lesion showing phagocytosis of damaged muscle fibres and fibrosis proceeding simultaneously. (*d*), Liquid paraffin (6 hr.). Slight cellular reaction around oil droplets; no damaged muscle fibres present. (*e*), Liquid paraffin (72 hr.). A considerable granuloma, in which eosinophil cells are prominent, has formed around the oil droplets. (*f*), Maize oil (72 hr.). Only a slight reaction to the oil droplets is seen.

Two hours after the injection of liquid paraffin little damage had occurred. The muscles surrounding the needle track appeared to have been pushed aside by the injected material, but only isolated fibres showed evidence of damage, presumably by trauma from the needle. There was no zone of oedema, and no evidence of cellular reaction. By 6 hr. rather more fibres showed evidence of damage, but it was still not extensive. The injection had now split into numerous isolated globules, which lay between, and distorted, individual muscle bundles without greatly damaging them. The oil droplets were surrounded by a little reaction with histiocytes and polymorphs in almost equal numbers (Fig. 2*d*). In these sections, and indeed in all the later sections from sites of injection of oily substances, it could be seen that there was a considerable tendency for the injec-

tion fluid to leak back along the needle track and into the subcutaneous tissues.

The muscle damage did not appear to increase during the next three days. The isolated globules of oil become well defined, by reason of a cuff of histiocytes and fibroblasts surrounding each individual globule. Even at 24 hr., polymorphs were no longer prominent in the cellular response; their place was taken apparently by eosinophils, which may be quite prominent in these granulomata. This prominence of eosinophils appeared especially marked in granulomata formed around globules of oil which had leaked back into the subcutaneous tissues. At 72 hr. each of these granulomata had, in addition to the eosinophils and histiocytes, a well-marked fibrous capsule (Fig. 2*e*).

The injection of maize oil gave rise to slightly more damage to muscle than did the injection of liquid paraffin, although the amount was still small compared with that found following the injection of even the less injurious aqueous solutions. The cellular response to maize oil was of the same general character as that to liquid paraffin, but it was much smaller in amount, nor was fibrosis at all prominent even at 72 hr. At this time, the individual globules were separated only by thin capsules consisting of a few fibroblasts with eosinophils and histiocytes (Fig. 2*f*).

Discussion

In man, purely local reactions to injected material may take the form of pain occurring immediately upon injection of the material, or very shortly thereafter, or of inflammatory reactions, sometimes with abscess formation occurring during the days following the injection. More rarely reactions remote in time from the injection may occur, for example the paraffin granuloma.

The mechanism of pain occurring immediately on injection is not clear. A possible explanation is that some substances may possess the property of causing excitation of nerve fibres and nerve endings with which they come into contact. Whatever the mechanism, no animal experiment to determine the probability of this event occurring in man readily suggests itself, and trial in man appears to be the only way of discovering whether this complication will occur.

If contamination by pathogenic organisms be excluded, inflammatory reactions occurring at an injection site must be due to the local effects of the injected material. Since, as has been shown above, even the most bland injection will cause some local damage, while local reactions sufficient to evoke clinical comment are not common with most injectable preparations, it is clear that a wide latitude exists in the damage that may occur at an injection site before a clinically evident condition manifests itself. This is fortunate, for, were this not so, therapy by intramuscular injection would be virtually impossible. It remains therefore to decide whether any parallel exists between effects produced by a substance in rat muscle on injection and the clinical effects of the same substance in man, and, if such a parallel exists, what amount of damage in the rat muscle represents the upper limit of clinical acceptability in man. Here it must be pointed out that clinical acceptability is not absolute, but depends on the purpose and efficacy of the medication; any local reaction no matter how severe would be acceptable in a reliable cure for cancer, while no reaction at all would be permissible in a cure for the common cold.

It is immediately clear that at least a rough parallel does exist between the rat test and results in man. Substances known to be bland on injection in man produce minimal local damage when injected into rat muscle. Normal saline and calcium gluconate exemplify this concordance. However, when substances differ markedly in the reaction produced in rat muscle as a consequence of their injection from the reaction consequent on the injection of saline there are no general grounds for predicting what level of reaction in rat muscle represents a clinically undesirable reaction even if the parallel between the two situations is always valid.

We have resolved this difficulty by the observation that all the substances known regularly to give rise to unfavourable clinical reactions, when tested in the rat, give rise to such necrosis of muscle that at 72 hr. necrotic muscle is still to be seen around which no reaction is occurring. It may also be that the frequency and severity of the clinical reactions are roughly related to the amount of such necrotic muscle. Quinine dihydrochloride is described above as an example of such a noxious injection. We have also found that, as might be expected, such substances give other evidence of their noxious properties. Thus, oedema is severe when necrosis is extensive, and the appearance of inflammatory cells is much delayed. Although this observation is empirical, it may perhaps be related to clinical experience more directly, since necrotic muscle present at three days, and not subject to phagocytic attack, must form a potential site for a sterile abscess due to autolysis of the dead muscle and the accumulation of necrotic inflammatory exudate, while dead

muscle fully phagocytosed in the first three days obviously does not represent so severe a hazard in this respect. It is worth noting that such residual dead muscle may also form a suitable nidus for bacterial growth.

It is thus our experience that, by this test, injectable materials can be divided into three categories: (*a*) Substances causing little more damage than normal saline. These may be expected to prove bland on injection in man. (*b*) Substances which in this test give a residue of necrotic muscle unattacked by phagocytes at 72 hr. These may be expected, in man, to give rise to severe local reactions at the injection site, often sufficient to preclude their use. (*c*) Substances giving rise to damage intermediate between (*a*) and (*b*). Prediction here is less certain, but examination of the histological appearances may suggest that the picture is close to one or other groups above, and the probability of clinical reaction may be correspondingly assessed.

Several factors must influence the local irritancy of injected materials, even when these are single pure substances in simple solution. Often the substance itself cannot, for therapeutic reasons, be appreciably modified. Our observations on distilled water, normal saline and hypertonic saline show that the tonicity of a solution modifies very strikingly the resultant muscle reaction. It is thus always advisable for injectable formulations to be as close to isotonicity as possible. This is not always efficacious in diminishing irritancy, as the observations on potassium penicillin demonstrate. This solution is approximately isotonic at 100,000 units/ml., but the irritancy of such a solution differs little from that of a solution of 500,000 units/ml.

Oils and oily solutions present a somewhat different problem from those discussed above. Few oils produce the immediate severe muscle damage seen with some aqueous solutions; however, the formation of a chronic progressive granuloma is a hazard, and this can be assessed equally well by the test we describe. We find it advisable to supplement the routine test with observations made at later periods following the injection. Nevertheless, even at 72 hr. it can be seen that the various oil granulomata differ greatly in size and activity. Our results are in agreement with those of Brown *et al.* (1944) in this respect and also when responses to vegetable oils are considered. A striking and constant feature of the oil granulomata seen in our rats with the oils described above, and with others not described here, is the eosinophil reaction. Eosinophils were noted by Story (1950) in an oil granuloma in man, although eosinophils were not noted in their animals by Brown *et al.* (1944). The significance of this phenomenon is not clear. It does not appear to be related to sensitization, and it can be evoked by oils of very diverse compositions.

Ultimately the only valid criterion of the clinical acceptability of an injection is, of course, trial in man. Before such a trial, it is desirable to have some idea of the likely behaviour of the material. Although many simple procedures could be developed to examine some aspect of the local irritancy of substances, none, we think, would model so closely conditions of use, and none would provide so reliable a basis for prediction of effects in man, as the technique described above.

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