

INTRANUCLEAR INCLUSION BODIES IN THE TISSUE REACTIONS
PRODUCED BY INJECTIONS OF CERTAIN FOREIGN
SUBSTANCES *

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In studies on prevention of experimental equine encephalomyelitis in guinea pigs by means of virus adsorbed on aluminum hydroxide Cox and Olitsky^{1, 2} observed in the phagocytic mononuclear and giant cells of the induced subcutaneous nodules intranuclear inclusion bodies characteristic of encephalomyelitis virus infection. When the chemical alone, free from virus, was introduced under the skin of guinea pigs, similar inclusions were seen in the resulting foreign body reaction. This finding gave a point of departure for the present investigation in which an attempt was made to study further the nature and significance of the nuclear changes brought about through the action of non-virus material.

Intranuclear inclusion bodies are found as characteristic changes in the lesions of many virus diseases.^{3, 4} In addition they have often been observed in tissues in which no search was made for the presence of virus although infection with virus was strongly suspected.^{5, 6} Cowdry,^{3, 4, 7} however, has stated that such structures are not necessarily the result of virus action and has held to the possibility that they may be produced experimentally by other means. A number of investigators, using non-infective materials, have produced bodies resembling the inclusions. It is noteworthy that in none of these procedures have attempts been made to eliminate the presence of virus occurring spontaneously in the animal tissues. (Summaries of and references to such reports are given in several articles.^{4, 8-12})

A wide variety of methods and materials was used in these recorded instances. Bodies which investigators or their later commentators believed to resemble virus inclusions were found in tissues moistened with ammonium chloride followed by applica-

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tions of direct electric current; in nerve cells repeatedly stimulated by electric current; in ganglionic cells soaked in hypertonic sodium and calcium chlorides; in rabbit corneas injected with bacterial toxins, and in pad tissues injured by heat or acid. Among other materials used were: hypertonic solutions of glucose, sodium chloride, and bicarbonate; bismuth, lead, arsenic, morphine, strychnine, salyrgan, exotoxins, and so on, given to animals by various routes of inoculation.

In certain of these reported cases the nuclear changes might be regarded as not conforming with inclusions typical of virus infection. In some the entire nucleus was reported as shrunken¹³; in others, suspected bodies could well have been degenerated nucleoli which stained pink instead of blue¹²; in still others the basophilic chromatin was clumped about the nucleolus,¹⁴ but in the remainder some resemblance to virus inclusions was apparent; for example, certain ones produced by Lee¹⁵ with salyrgan were, in Cowdry's opinion,⁴ indistinguishable from structures produced by viruses. As already mentioned, however, in none of these cases have attempts been made to exclude the possibility of a preexisting or concomitant virus as the causal factor in the production of the structures.

The following criteria are generally considered as the characteristic features of true virus inclusions. Their morphology must, of course, be based on studies of structures found only in virus-containing tissues; that of bodies which have no proved relationship with any virus is not applicable. By many investigations on undoubted virus inclusions it has evolved that no single characteristic feature is invariably found but that three particular properties are of outstanding importance, namely, (1) acidophilic staining, (2) halo formation, and (3) margination of chromatin with or without displacement of nucleoli. Cowdry^{7, 16} has attempted to define the nuclear inclusions more precisely and to obtain clues of their chemical constitution by the use of histochemical reactions. Most noteworthy of these are the Macallum test for masked iron and the Feulgen reaction for thymonucleic acid; both give positive reactions with nuclear chromatin and negative results with virus inclusions.

Nuclear inclusions have also been classified in two types by Cowdry. Type A includes generally bodies in nuclei in which all

of their basophilic chromatin is ultimately marginated; such are found in herpes, yellow fever, virus III disease, pseudorabies and others. In Type B the basophilic chromatin fails to marginate and the acidophilic bodies appear in various parts of the nucleus; they occur in poliomyelitis, Bornu's disease, equine encephalomyelitis, and others. Both types may, however, occur in the same disease, as shown by Cowdry,⁴ Sabin and Hurst,¹⁷ and by our own observations.

The general characteristics which are commonly ascribed to proved virus inclusions have been mentioned but it is known that variations occur. Most specific intranuclear inclusions are acidophilic but some have been described¹⁷⁻¹⁹ that are either partially or completely basophilic. Presence of halos depends on the particular virus and the stage of infection. In herpes²⁰ halos appear only in later stages. Margination occurs irregularly; Cowdry, Lucas and Fox²¹ have noted it in normal cells and we have observed it in nuclei without inclusions. The histochemical reactions have not been universally applied.

METHODS AND MATERIALS

Aluminum hydroxide was prepared essentially according to the directions for making Willstätter's Type C gel.²² This was suspended in $\frac{M}{30}$ phosphate buffer at pH 6.6. Another preparation was secured by adding equal parts of ammonium hydroxide (C. P., 28 per cent NH_3) and a solution of 32 per cent aluminum sulphate with shaking at room temperature. The resulting precipitate was washed with distilled water until free of ammonia, using the centrifuge for sedimentation. A third form of aluminum consisted of alundum, an insoluble form of aluminum oxide commonly used as an abrasive in grinding virus-containing tissues. A weighed amount was thoroughly ground in a mortar with distilled water until a fine suspension resulted. Ferric hydroxide was obtained by adding ammonium hydroxide to a clear solution of ferric chloride and washing in the same way as the Type C gel. Suspensions of barium sulphate, silver chloride and carbon were prepared by adding distilled water to the required amounts. Parowax (a paraffin of moderately low melting point) and 2 per cent nutrient

agar with Witte's peptone were also used. All preparations were autoclaved.

Materials were injected under the skin in guinea pigs and rabbits in amounts of 1 cc. in each site. The object was to produce palpable nodules that could be removed at various intervals of time. It was found that size and persistence of nodules depended mainly on concentration and composition of the material, the depth of injection, and the thickness of the skin. Those of aluminum hydroxide usually lasted 6 to 8 weeks. Ordinarily 7.5 to 10 mg. per cc. of aluminum oxide or of the other chemicals were used but concentrations as high as 20 to 60 mg. per cc. of ferric hydroxide, barium sulphate, or carbon were sometimes necessary to bring about persistent nodules. Paraffin was given undiluted. When the first injection did not result in the formation of nodules, the same sites were reinjected 2 or 3 days later with the same or a higher concentration of the same material. The time of removal of nodules was then counted from the date of second injection.

Nodules were excised* aseptically at various intervals. The skin was closed by clips and small dressings of gauze with colloidion were applied. In this way 4 or 5 nodules could be removed from a single animal. The excised tissue was cut so that a piece of skin was retained and then fixed in Zenker's solution containing 5 per cent glacial acetic acid in preparation for ordinary stains to be mentioned, and in special fixatives for certain other stains, as shown in Table II.

TYPES OF TISSUE REACTIONS

During the first few days after subcutaneous inoculation of the mentioned foreign substances in guinea pigs (and in the case of aluminum compounds and of ferric hydroxide, in rabbits), the tissues at the site of injection responded with the usual type of inflammation — chiefly polymorphonuclear cell invasion, edema and congestion. After 4 or 5 days the individual reaction to each of the substances became manifest.

Table I summarizes the types of induced reactions. With the aluminum substances, the hydroxide C gel or ordinary form, or alundum, a foreign body nodule was brought about. The central

* Operations on animals were done with the aid of ether anesthesia.

portion contained heterogeneous material of broken down cells, the injected chemical itself, débris, and a few polymorphonuclear

TABLE I
Substances Used for Injection and Their Effects in Guinea Pigs

Substance	Main features of tissue reaction 1 week after inoculation	Presence of inclusions
Aluminum hydroxide (Willstätter's Type C)	Phagocytic mononuclear cells and giant cells	+ +*
Ordinary aluminum hydroxide	Same	+ +
Alundum (Al ₂ O ₃)	Same	+ +
Ferric hydroxide	Same	+
Barium sulphate	Same	-
Silver chloride	"Cold" abscess surrounded by newly formed connective tissue. No giant cells	-
Paraffin	Strands of fibroblasts surrounding and penetrating the paraffin. Few phagocytic mononuclear and giant cells	-
Carbon	Phagocytic mononuclear cells and giant cells	+
Agar	Same	-

* + + indicates that numerous inclusions occurred from about the 7th day after inoculation and then persisted throughout the duration of the lesion (for several weeks).

+ indicates that they occurred only temporarily and in small numbers.

and monocytic leukocytes. This was surrounded by a dense mass of phagocytic mononuclear and later of giant cells (Figs. 1 to 6).* A connective tissue band of varying width was formed at the outer

* With certain chemicals (aluminum, iron) the giant cells contained nuclei arranged peripherally; with others (barium, paraffin, and carbon), these cells exhibited nuclei that were scattered throughout the cytoplasm, and with agar both forms, especially the former, were produced. The foreign body was, however, engulfed by the cytoplasm of both types of giant cells. Supravital and Masson's stains were performed only on the aluminum material. Here the precise classification of cells comprising the nodular lesion could be made (with the assistance of Dr. A. L. Joyner) and revealed phagocytic mononuclear, stimulated phagocytic mononuclear, epithelioid, and Langhans' giant cells (those with peripherally arranged nuclei).

margin. The phagocytic mononuclear and giant cells progressively invaded the central material from its periphery and engulfed most of it. The process continued until all miscellaneous contents of the center were removed, ultimately to end in healing by scar tissue formation. The lesions induced by ferric hydroxide were similar. The iron in stained or unstained nodules could be easily identified by its pronounced brown color both in cytoplasm and in the central area (Fig. 7). Barium sulphate after inoculation gave rise to nodular formation, the principal cells in which were phagocytic mononuclear and giant cells. The chemical could be seen both in the cytoplasm of these cells and in the core of the lesion as colorless, ice-like crystals. Silver chloride produced a different kind of lesion — a “cold” abscess of polymorphonuclear cells surrounded by a band of newly formed connective tissue cells, terminating finally in fibrosis. No giant cells were present. With paraffin were found strands of fibroblasts surrounding and penetrating the substance which was not seen in sections since solvents used in preparation had removed it. The lesion also contained a few phagocytic mononuclear and giant cells. Carbon induced localized areas of these cells surrounded by a connective tissue membrane. The black carbon particles were found to be packed into the cytoplasm of both forms of cells. Agar brought about nodules²³ which consisted of these cells with a thin layer of fibroblastic tissue enclosing the lesion. In earlier stages (to 7 days) agar as thin acidophilic strands was noted in the center and as amorphous material in the cytoplasm; later, the agar disappeared except for small, indefinite cytoplasmic masses.

To summarize, there were found in the lesions induced by agar and the chemical substances, except silver chloride, a preponderance of phagocytic mononuclear and giant cells. In those cells in which the cytoplasm visibly engulfed the foreign body, no trace of their presence could be seen in the nucleus (Figs. 3 to 7). The apparent failure to penetrate the nucleus when the cytoplasm was packed to the bursting point by the injected material should be stressed since a problem on the source of any intranuclear inclusions must first take into consideration the possibility of intranuclear entry of particles of the foreign body itself. This negative finding, taken together with others to be described, points to the fact that such invasion did not occur.

INCLUSION BODIES

Location and Morphology: Inclusions were present only in the nuclei of the phagocytic mononuclear and giant cells which were produced by aluminum compounds, ferric hydroxide, and carbon, and not in the nuclei in the lesions induced by the other materials. In the aluminum nodules they appeared in small numbers about 7 days after injection, that is, a short time after the phagocytic mononuclear cells were apparent in large numbers. They increased progressively until at about the 24th day there were often 10 to 12 per oil immersion field. Nodules from different animals, however, showed some variation in numbers. How long they persisted depended on the duration of the nodule. Inclusions were first seen with ferric hydroxide on about the 18th day and then only in half the number of animals. At about the 24th day they were not demonstrable. However, when present they were quite numerous. With carbon the acidophilic intranuclear bodies were observed first on the 18th day after inoculation, then again on the 24th day. Thereafter the lesion, as formed by the described method, disappeared. In numbers the bodies were less than in the aluminum material and roughly equaled those found in the iron preparations.

Although the aluminum compounds and ferric hydroxide produced the same reaction in rabbits, yet the inclusions were extremely rare or absent in this animal. This was in contrast with guinea pigs in which they were invariably found after the first 7 days. As a corollary to the presence of inclusions in one species of animal and not in another, reference should be made to the work of Hurst¹⁸ who could not demonstrate intranuclear inclusions in pigs infected with pseudorabies virus, although they were present in other animals, such as the rabbit, guinea pig, monkey and cow.

In stained preparations in the instances in which aluminum was injected, the contrast between the appearance of nuclei containing inclusions and those free from these bodies was striking. Non-inclusion-bearing nuclei were regularly basophilic. Such a nucleus consisted of a round or ovoid nuclear membrane within which were usually one or more nucleoli of various sizes and shapes. In addition, other more finely divided basophilic material was scat-

tered throughout. Minute amounts of acidophilic substance were also rarely seen but were not considered to be inclusions because of their small size, their lack of form, and their occurrence in otherwise normal cells. Amphinucleoli (basophilic nucleoli with acidophilic cores) were not ordinarily present. Basophilic chromatin was occasionally found collected on the nuclear membrane making it appear thicker and comparable to the margination of inclusion-bearing nuclei.

A constant characteristic of the inclusions was their property of acidophilic staining and this was used throughout as the most important criterion for their recognition (Figs. 3 to 7). No structure was thought to be an inclusion that did not stain with phloxine or some other "acid" dye and this made it necessary that differentiation be carried out properly during the staining procedure. Unless the nucleoli of the cells of adjacent tissues were definitely basophilic, the preparation was not considered suitable.

Most of the acidophilic bodies were approximately equal in size to average nucleoli. Others were larger, however, and some nearly filled the nucleus and could be seen without an oil immersion lens (Fig. 2). Great variation was observed with regard to form. Round, flat, whole-edged ones were common; some were angular, others fluffy. A number of them appeared to be composed of minute granules. Ordinarily they appeared less dense than nucleoli and more refractile (Fig. 2). As many as four were found in some nuclei, although one or two was the usual number.

In most instances margination of chromatin occurred, the nucleoli and other basophilic material being found collected peripherally on the nuclear membrane. Clear spaces or halos were usually to be seen around the inclusions. Nuclei containing the bodies at times were slightly larger than their fellows and appeared as a rule as clear sacs in which the ground substance could not be seen.

From their general appearance and also from their morphological characteristics it would seem that the inclusions produced by the substances mentioned are not distinguishable from those that occur in virus-containing tissues. Variations in form occur so that ones corresponding to either of Cowdry's types have been found in a single preparation. From the results of tests to be described immediately, additional similarity can be shown to exist between the chemically induced inclusions and those of virus diseases.

Tinctorial and Histochemical Tests: The purpose of these tests was to determine whether the intranuclear inclusions induced by the action of aluminum and iron compounds were (1) identical in their reaction to those brought about by viruses, (2) merely particles of the introduced foreign bodies, or (3) degenerated nucleoli.

Most of these tests were performed on nodules resulting from injection of aluminum compounds since these showed uniformly formation of abundant characteristic intranuclear inclusions. In Table II will be found a summary of the reactions of the inclusion, the nuclear structures, and the aluminum material engulfed in the cytoplasm.

It will be noted that the inclusions evoked by means of chemicals maintain their acidophilic character with different stains, as is the case with those brought about by viruses. The only exception is iron hematoxylin. With the same stain, however, Sabin and Hurst¹⁷ found the inclusions of B virus to be occasionally basophilic. Moreover, with most of the stains, color differences could be seen between inclusions and the aluminum hydroxide present in the center of the nodule or the cytoplasm of the cell. This suggested that the inclusions were not composed of aluminum hydroxide but did not exclude the possibility that within the nucleus it might have a different color because of combination or adsorption with organic substance there. However, as we have already stated, it was apparent that of all the chemical substances and agar injected, none was found by microscopic examination to have penetrated the nucleus.

The next series of tests concerned the possibility of identifying the inclusions with nuclear material. The Feulgen reaction is a test that has been widely used to detect the presence of thymonucleic acid which is a component of nuclear substance. Cowdry^{7, 16} has found that the intranuclear inclusions of certain virus diseases do not give the reaction. It was applied to the inclusions under investigation following his method, except that in addition counterstains of eosin or orange G were used. Like those of virus diseases, these inclusions gave a negative reaction in contrast with the other parts of the nucleus and so presumably did not contain thymonucleic acid.

TABLE II
*Staining Reactions of Intranuclear Inclusions in Nodules Produced by
 Aluminum Hydroxide*

Stain, counterstain	Fixative	Inclusions	Nuclear membrane and nucleoli	Aluminum hydroxide in cytoplasm
Methylene blue, phloxine	Zenker's (5% acetic acid)	Red	Dark blue	Lavender or unstained
Methylene blue, eosin	Zenker's (5% acetic acid)	Pink or red	Dark blue	Lavender or unstained
Hematoxylin, eosin	Zenker's (5% acetic acid)	Dark red	Blue-black	Lavender or unstained
Hematoxylin, orange G	Zenker's (5% acetic acid)	Gray	Dark blue	Light yellow or unstained
Giemsa	95% alcohol	Dark red	Dark blue	Gray, laven- der, or un- stained
Methyl blue, eosin (modified Mann)	Zenker's (5% acetic acid)	Red	Blue	Lavender or unstained
Iron hematoxylin, orange G	Zenker's (5% acetic acid)	Black	Black	Yellow
Masson's connec- tive tissue stain	Helly's fluid	Dull brown or dark gray	Brown	Bright green
Feulgen, eosin	95% alcohol or sublimite alcohol	Red	Purple	Light pink or unstained
Feulgen, orange G*	95% alcohol	Orange	Purple	Light yellow or unstained
Masked iron, eosin	95% alcohol	Dull red	Blue-black	Lavender or unstained
Masked iron, orange G	95% alcohol	Yellowish gray	Blue-black	Light yellow or unstained

* This counterstain was used as recommended by J. A. de Tomasi (*Stain Technology*, 1936, 11, 137).

Cowdry¹⁶ has also found that the virus inclusions he studied reacted negatively to the Macallum test for masked iron. This is a test for iron in organic combination such as is present in the basophilic chromatin of normal nuclear material. The procedure was carried out essentially according to the directions of Nicholson²⁴ and Lee.²⁵ Sections were first allowed to stand in a solution of 4 per cent sulphuric acid in order to "unmask" the iron, that is, change it into inorganic form. This was followed by a yellow solution of pure crystalline hematoxylin * which combined with this iron and gave a blue color. A counterstain of eosin or orange G was then applied. Again like those of virus diseases, these inclusions gave a negative reaction for masked iron.

Preliminary work with Zenker-fixed tissue on the application of the test for masked iron to nodules of ferric hydroxide showed that this substance itself did not react but retained a brownish yellow color similar to that in sections stained by ordinary methods. It was therefore considered unlikely that this method could give conclusive evidence as to whether ferric hydroxide had appeared within the nucleus modified in such a way as to stain as inclusions. However, such a possibility seemed remote in view of the fact that this iron compound showed such a distinctive color and apparently nowhere penetrated the nucleus.

The method of microincineration has also been applied to determine the mineral content of several virus inclusions.^{20, 26-28} The procedure was employed in the present study essentially as described by Scott.²⁸ With the material studied, the results were disappointing since the nodules contained quantities of inorganic substance which left an abundant ash after the organic material was burned away. The ash left by the aluminum hydroxide covered the whole area in which inclusion-bearing nuclei were to be sought, thus obscuring the fields under observation.

Other Tests: Sodium alizarin monosulphonate added to salts of aluminum gives a red color; this reaction offers a delicate test for this element. Attempts were made to develop a histochemical test using this dye with the idea of detecting the presence of

* When the preliminary treatment with acid was omitted from control sections the nuclei were not colored with this solution. 95 per cent alcohol was used for fixation because when such a control was used with sections of tissue fixed in Zenker's solution the nuclei gave a non-specific reaction.

aluminum in the inclusions. All such trials have ended in failure because the aluminum hydroxide in the center of the nodule or in the cytoplasm would itself never give a positive test. It is a very insoluble substance and any procedure designed to bring it into solution has a deleterious effect on the organic structures in the section. That aluminum was still present at the times nodules were removed was shown by gross chemical tests.

The method of supravital staining was tried on inclusion-bearing nodules. One could see the nucleolus eccentrically placed in the nucleus and suggestions of margination of nuclear material. Moreover, in some of such nuclei, bodies lighter in texture, more homogeneous, and resembling inclusions were seen. However, it could not be said with certainty that these were the same structures that were seen in fixed preparations.

From the results of these tests it would seem that the intranuclear inclusions evoked by the injection subcutaneously in guinea pigs of the aluminum and iron compounds have many resemblances to virus inclusions. The similarity exists not only in morphology and location but in the response of the bodies to the Feulgen and masked iron tests. Furthermore, the recorded reactions point to the fact that the inclusions under investigation are not merely degenerated nucleoli or artefacts due to technical procedures, and finally that they do not in themselves represent particles of injected chemicals.

SEARCH FOR VIRUS

The presence of inclusions suggested the action of a virus (1) preexisting in the animals and activated by the injected materials; (2) "formed *de novo*" as questioned by Cowdry^{3,7} as a possible occurrence if intranuclear inclusions were produced by artificial means; (3) being a contaminant, perhaps one of the viruses studied in this laboratory, and (4) carried along with the materials used. The fourth can be eliminated by the fact that such substances were autoclaved. Because of the other three possibilities, evidence of a virus was sought. An outstanding example of where the finding of intranuclear inclusions led to the discovery of a virus is that afforded by the work of Cole and Kuttner²⁹ on the salivary gland virus of guinea pigs. This infective agent is ap-

parently present in most of the guinea pigs of certain stocks without giving rise to symptoms but is, nevertheless, associated with intranuclear inclusions. Such inclusion-containing material is, however, capable of transmission in series to young guinea pigs which then show the clinical disease with death.

Transmission Experiments: The purpose was to select animals believed to be most susceptible and to inoculate into them by various routes heavy suspensions of inclusion-containing tissue. Nodules resulting from injection of aluminum compounds were used as source material. As we have already mentioned, these compounds formed numerous inclusions regularly.

Nodules were removed at intervals of 3, 7, 10, 12, 17 and 24 days. These were ground aseptically with alundum and enough sterile Tyrode's solution to make dilutions from 1:5 to 1:10. After light centrifugation to deposit gross particles including the alundum, the supernatant liquid was used for inoculation. All the injected animals and the original ones bearing nodules were kept in a separate room and isolation precautions were taken. When the nodules were removed, part was taken for section so that it could be determined whether the material actually used for passage contained inclusions. All of these nodules, except the one removed at 3 days, did show inclusions.

A total of 36 mice was given intracerebral injections of 0.03 cc. each of such material. One died of the inoculation; the rest survived for 1 to 2 weeks and during this time showed no clinical evidence of disease. The brains of 3 of these mice were removed for section after 7 to 12 days of observation but no lesions attributable to the action of a virus were found and no inclusions seen.

Fifteen young guinea pigs (10 to 12 days old) were given intracerebral inoculations of 0.05 cc. each. In addition, 3 of them received subcutaneous doses of 1 cc. One died of a perforated colon, and the brains of 4 others were removed for section on the 12th to 15th days. No lesions or inclusions were found. The remaining ones were observed for 2 to 3 weeks but in none did clinical signs of disease develop.

Twelve adult guinea pigs were each given intracerebral inoculations in doses of 0.1 cc., subcutaneous inoculations in doses of 1 cc., and intradermal and subcutaneous inoculations in a pad. The brains of 4 of these were removed for section at intervals of 2 to

12 days. The pads of 6 were removed for section at intervals of 2 to 12 days. Neither brains nor pads showed lesions or inclusions. Barely perceptible transitory indurations appeared in 6 of the animals at the sites of subcutaneous injection. No reactions whatever occurred in others in which the inoculum had been centrifuged more strongly. Skin and subcutaneous tissue at the site of injection were removed for section at intervals of 2 to 12 days from 6 animals. Microscopically the indurations consisted of minute collections of phagocytic mononuclear cells. In 2 of the sections occasional inclusions were found. In these cases tissue had been removed 12 days after subcutaneous inoculation. It was believed that in the instances in which minute indurations were produced these areas contained sufficient chemical which was carried along with the inoculum and set up a slight transient reaction. None of the adult guinea pigs showed clinical signs of infection.

Tissues of animals that had been injected with ground nodule in attempts at transmission of virus were, in turn, ground again and injected into other guinea pigs, thus effecting a second "passage." In 1 test, 2 of the minute, subcutaneous indurations described above were ground with alundum and Tyrode's solution. The supernatant fluid after centrifugation was injected subcutaneously into 6 adult guinea pigs in doses of 1 cc. Four of the 6 developed almost imperceptible indurations that disappeared in about 1 week and the sites of 2 of these were removed at 12 and 13 days for section but no lesion other than some increase in fibroblastic tissue was seen. In another test, brains of 4 young guinea pigs which had received intracerebral injections were ground with alundum and sterile Tyrode's solution. After centrifugation they were injected intracerebrally into 6 adult guinea pigs in doses of 0.1 cc. No signs of infection resulted.

It is therefore apparent from the tests performed on mice and guinea pigs that were inoculated with nodular tissues containing inclusions that no evidence of virus infection was obtained.

Reinoculation Test: One might assume that the association of a possible virus with the nodules might set up an immunity so that subsequent injections of aluminum hydroxide would not produce inclusions. An experiment was planned to test this point.

Five guinea pigs were each given 3 inoculations of aluminum hydroxide. One nodule was removed for section from each in 12

to 30 days; these were found to contain the characteristic intranuclear inclusions. On the 41st to 56th day after first injection these animals and a normal control were reinoculated. Twelve or 24 days later nodules derived from the reinjection were removed for section. In addition, some of the first nodules, now 53 days old, were examined.

Inclusions were found in all of the sections of old and new nodules. Consequently no immunity, as postulated, could be shown to exist, since typical new intranuclear structures could be produced, regardless of whether others were present at the time or had previously been demonstrated in the same animals.

Examination of Submaxillary Glands: In view of the fact that the inclusions were observed in guinea pigs but not in rabbits and that adult guinea pigs are known to be infected often spontaneously with salivary gland virus, an attempt was made to obtain evidence of its presence. In addition to the transmission experiments already described, this was done by examining for virus inclusions the submaxillary glands of guinea pigs in which nodules produced by aluminum hydroxide had shown inclusions.

Right and left submaxillary glands were removed for section from 4 adult guinea pigs in which the nodules formed by means of aluminum hydroxide had shown many inclusions. The tissues of 2 of these animals revealed occasional intranuclear inclusions typical of the salivary gland virus. The remaining 2 showed none. Two young (10 day old) guinea pigs were given injections of aluminium hydroxide. Nodules were removed on the 7th and 18th days afterwards and also on the latter date (28th day of life) the right and left submaxillary glands were excised for study. Inclusions were found in all 4 of the nodules but in none of the submaxillary glands.

Reference is also made to the fact that in the transmission experiments to young guinea pigs with nodular material, no infection with salivary gland virus was discernible in inoculated animals. Moreover, the characteristic basophilic marginal structures surrounding the intranuclear acidophilic inclusion body of salivary gland disease was not generally seen in the nodules induced by aluminum compounds. It is therefore not likely that the intranuclear inclusions found in the latter represent the bodies brought about by this virus.

In the foregoing experiments attempts were made to detect the presence of any virus that might possibly have been the causal factor in the production of the described intranuclear inclusion bodies. These trials were made by means of animal inoculation of affected tissues, by tests to show whether immunity was induced, and by histopathological studies. What were believed to be the most favorable routes of inoculation and the most susceptible animals were employed. From the results it is apparent that a virus was not demonstrated under these experimental conditions.

DISCUSSION AND SUMMARY

In the present paper is shown the formation of intranuclear inclusion bodies by the subcutaneous injection of guinea pigs with different aluminum compounds, less often with ferric hydroxide and carbon. They appear to resemble virus inclusions so closely as to be practically indistinguishable from them. One might add that in the instances in which ferric hydroxide and carbon were used they appeared after a prolonged interval and then only for a short period during the height of the reaction. Similar occurrences are known in virus pathology. With aluminum compounds, after the initial period of about 7 days passed, they were observed throughout the duration of the lesion. Finally, special types of cells, phagocytic mononuclear and giant cells, only are involved, and not all foreign body tissue reactions contain the inclusions — barium sulphate, silver chloride, agar, and paraffin have failed.*

The mechanism underlying the production of these chemically induced inclusions is unknown. Just as with virus inclusions they fail to give the Feulgen (thymonucleic acid) and the masked iron reactions; this is taken as evidence that they do not originate from degenerated nucleoli. Another source might be particles of the introduced foreign substances. In this connection Jacobs,³⁰ in an article on cell permeability, warns against assuming that a colored substance has not penetrated a cell merely because it cannot be seen.³¹ While nuclei are being dealt with and not cells as a whole, a

* No inference is intended that the aluminum and iron compounds and carbon are the only substances that can possibly give rise to inclusions.

conclusion cannot be reached on this basis as to whether or not the inclusions within the nuclei are particles of the injected substances. This possibility is remote, however, in view of the visible material used, and since in no instance could any part of it be observed to have entered the nucleus.

The point to be stressed is that the intranuclear inclusions produced by chemical means are apparently not associated with a virus; a search which included a number of transmission experiments to animals of the same species in which they were originally produced gave a negative result. In view of this, it is evident that no disease can be classified as a virus disease on evidence of finding in pathological examination of tissues intranuclear inclusion bodies unless it can be proved that they are associated with a virus, that is, unless experimental transmission can be effected.*

While intracytoplasmic inclusions occurring in the lesions set up by viruses are generally considered as colonies of virus particles — they are commonly found associated with such infective agents of larger size — it becomes more difficult to interpret the significance of virus intranuclear inclusions. One interpretation offered is that changes called forth in the cytoplasm due to virus action might possibly induce changes in the nucleus with the accompanying formation of the intranuclear bodies. From what is here reported, it would appear that a similar sequence of events might follow the engulfing of foreign bodies by cytoplasm, which in turn might influence the nucleus in the manner already described. It is, however, unknown whether or not the structures within the nucleus that appear to resemble so closely those associated with viruses are identical with them in their physicochemical natures.

CONCLUSIONS

1. Intranuclear inclusion bodies resembling in certain important ways those found in virus diseases were induced by chemical substances such as selected aluminum and ferric compounds and

* Alundum is used commonly as an aid in grinding virus tissues. It has been shown that alundum itself may induce localized tissue reactions associated with virus-like inclusion bodies. It is therefore conceivable that such inclusions might be mistaken for bodies induced by viruses if care is not taken to centrifuge the alundum out of tissue suspensions to be injected.

carbon but not by others such as barium sulphate, silver chloride, and paraffin, nor by agar.*

2. No evidence was revealed of a virus infection in association with these inclusions.

3. The results throw light on the interpretation of intranuclear inclusions as they occur in lesions caused by viruses.

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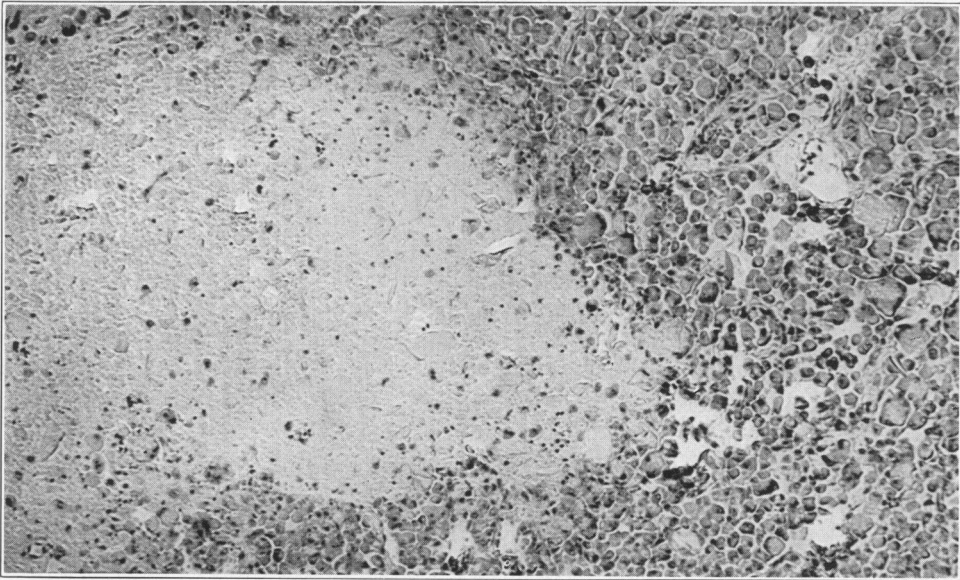
* After this paper was submitted for publication, intranuclear inclusion bodies similar to those described here were seen in subcutaneous nodules induced in guinea pigs by injection of brain tissue derived from apparently normal animals. This aspect of the problem is now being studied.

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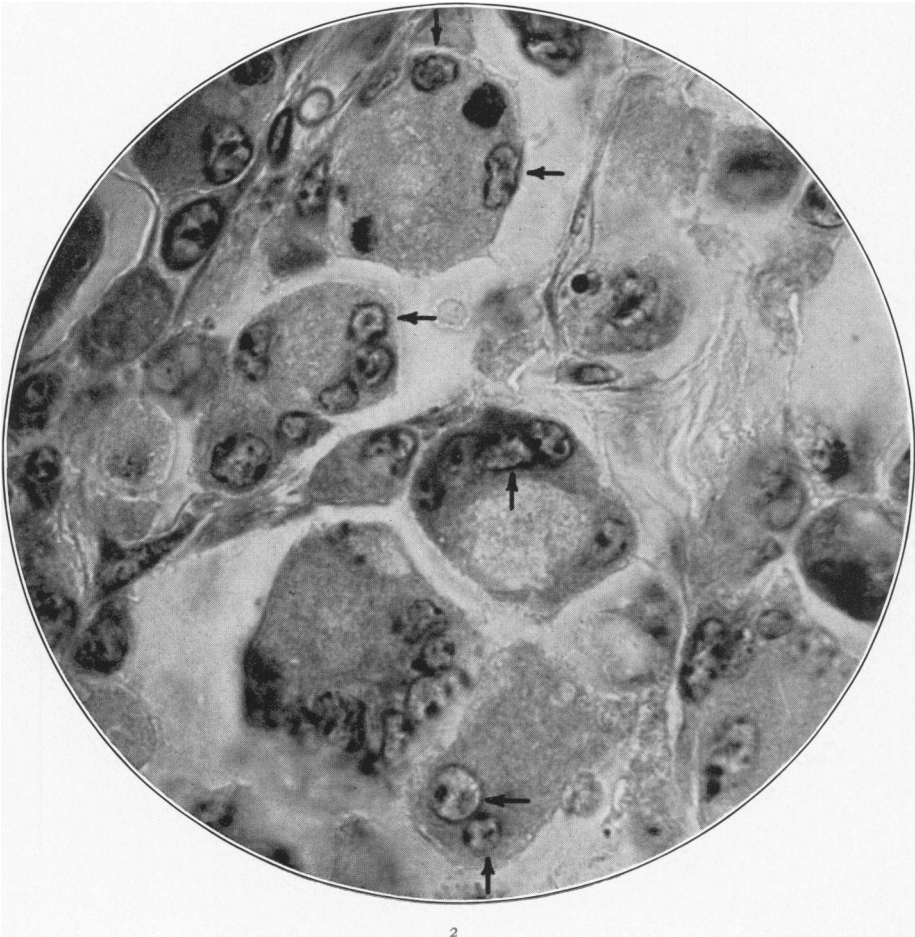
DESCRIPTION OF PLATES

PLATE 102

- FIG. 1.** Section of a nodule produced by aluminum hydroxide removed from a guinea pig 24 days after injection. The tissue reaction of phagocytic mononuclear and giant cells is shown about the central portion of tissue detritus and chemical. Phloxine-methylene blue. $\times 100$.
- FIG. 2.** Same section as Fig. 1. Several epithelioid giant cells with peripherally arranged nuclei are present in this field. Arrows point to inclusions. Note that these bodies appear less dense than the nucleoli in their own and adjacent nuclei. Uppermost arrow indicates a large, herpes-like inclusion. Phloxine-methylene blue. $\times 1000$.



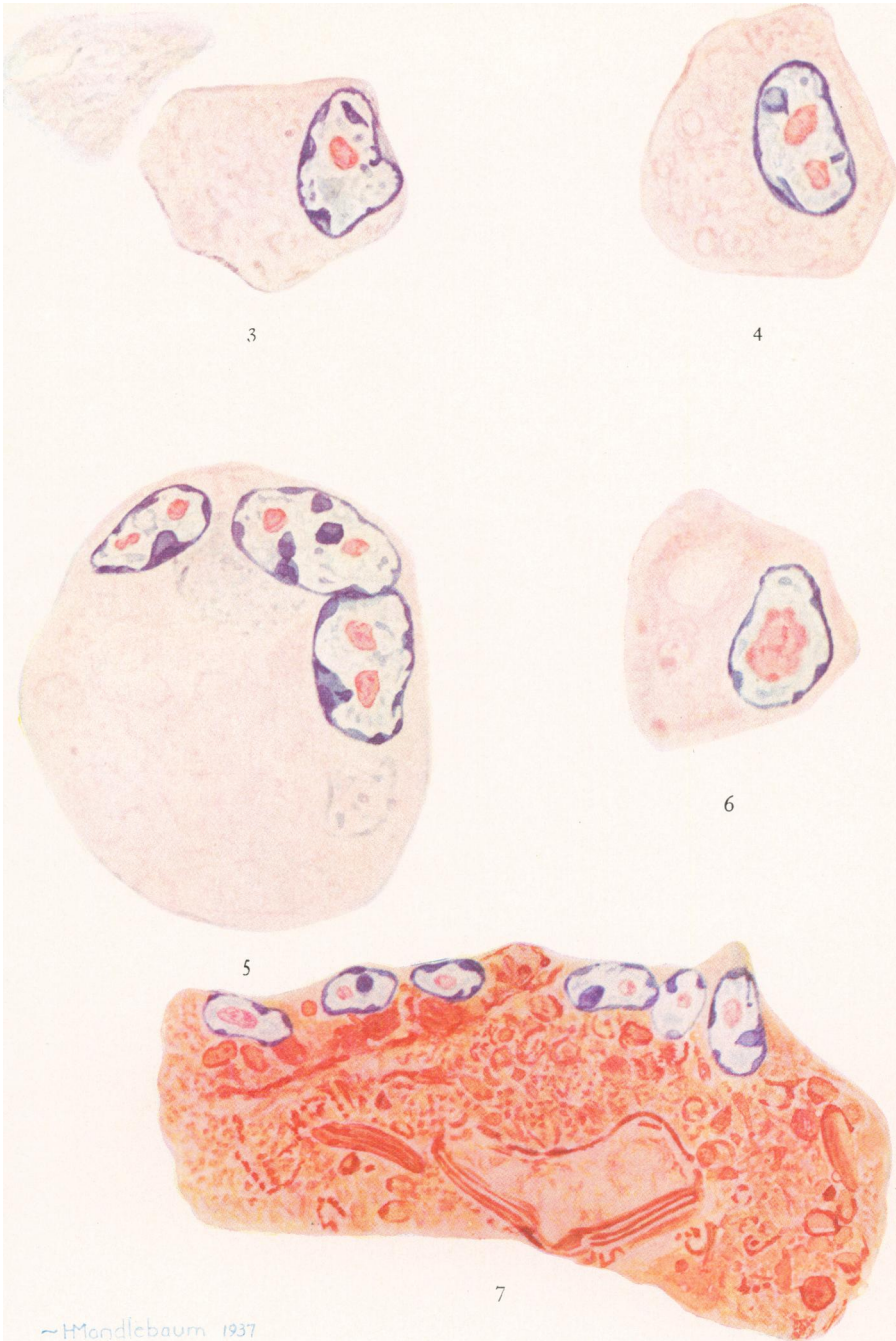
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PLATE 103

- Fig. 3. Common variety of an inclusion, a single body with margination of the chromatin. In the upper left corner is some amorphous debris and aluminum hydroxide found in the center of a nodule. Phloxine-methylene blue. $\times 2100$.
- FIG. 4. A nucleus containing two inclusions. Phloxine-methylene blue. $\times 2100$.
- FIG. 5. Giant cell showing inclusions in all of 3 nuclei; 2 other nuclei are shown in shadow because of their deeper sites in the cell. Phloxine-methylene blue. $\times 2100$.
- FIG. 6. Large type of inclusion in nodule produced by aluminum hydroxide. Phloxine-methylene blue. $\times 2100$.
- FIG. 7. Giant cell in a reaction produced by ferric hydroxide in a guinea pig 18 days after injection. The compound of iron is recognized by its brown color. It distends the cytoplasm but apparently does not penetrate the nuclei. Some of the latter are flattened at the periphery of the cell and contain inclusions which stain red with phloxine. In these nuclei also may be seen several degrees of margination of the blue staining chromatin. Phloxine-methylene blue. $\times 1500$.



Olitsky and Harford

Intranuclear Inclusion Bodies