THE PATHOGENIC AGENT IN NORMAL HUMAN BONE MARROW

ITS NATURE AND RELATIONSHIP TO THE LYMPHADENOMA AGENT OF GORDON

BY

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In a previous paper¹ it was shown that when normal human bone marrow is injected intrathecally in rabbits these animals develop a progressive and fatal disturbance of the nervous system, paralysis of the hind legs being a characteristic feature of the late stages. It was recognized that the clinical appearances presented by these rabbits resembled those which M. H. Gordon² had already found in rabbits injected intracerebrally with material derived from human cases of lymphadenoma, but the relationship of the two pathogenic agents was not more closely determined. The nature of the agent contained in normal human bone marrow—whether it is a living or a non-living thing—was also left in doubt. The primary purpose of the present paper is to describe experiments directed towards the solution of these two problems.

Right at the outset I must acknowledge deep indebtedness to Dr. Gordon. To his efforts and kindness I owe all the samples of human bone marrow and viscera used in the series of experiments to be described. I have called these human tissues normal in the present paper. This is because I am satisfied they have represented the conditions present in normal human beings. Actually the specimens have been obtained post mortem from a large variety of pathological conditions and from cases of accidental death.

NATURE AND DISTRIBUTION OF THE PATHOGENIC AGENT

The living nature of the pathogenic agent present in normal human bone marrow would be satisfactorily established if this agent could be passed from rabbit to rabbit indefinitely. Renewed attempts to demonstrate such a passage have failed. Intracerebral inoculation of brain tissue taken from a paralysed rabbit was on one occasion followed by somewhat suggestive symptoms. This is the nearest approach to a positive result that I have seen in the new series of experiments. In the previous paper it was reported that in one experiment there had been evidence of transmission through three generations in rabbits, but we have not seen the like again. Perhaps it is significant that in this apparently successful experiment the first rabbit of the series was injected with bone marrow from a case of pernicious anaemia, but we cannot exclude the possibility that some adventitious virus may have been present. As the facts stand it has to be realized that serial transmission of the bone-marrow agent has not been proved, and that in this direction attempts to show the viral nature of the agent have broken down. Such failure is, however, not very strong evidence for the non-living nature of the agent.

It was thought that some knowledge of the species and organ distribution of the agent might be useful. In numerous experiments intrathecal and intracerebral injection of rabbits has shown that the pathogenic agent under investigation is not present in normal bone marrow of rabbits, guinea-pigs, cats, and horses. The bone marrow of dogs has not been tested: for technical reasons I found it difficult to obtain suitable material. The spleen and leucocytes of normal dogs, however, have been tested a number of times, and have given consistently negative results. Normal human liver and kidney also gave negative results, but normal human spleen produced symptoms similar to those produced by normal human bone marrow, though not with the same regularity. Difficulties arising

from the fact that available samples of human viscera were seldom free from contaminating micro-organisms disappeared when the material to be used in the experiments was heated for one hour at 65° C.

The consistency with which bone marrow from so many species has given negative results, and the fact that human liver and kidney are also innocuous, disposes finally of the possibility, discussed in the previous paper, that human bone marrow acts simply by stirring up some virus normally present in the rabbit's brain. The histological changes which follow the intrathecal and intracerebral injection of normal human bone marrow and spleen have not been investigated. For want of some better term we spoke of the condition as an encephalitis in the previous paper, and may do so again in this, but I do not wish to be misunderstood: the term is not used to indicate an opinion as to the ultimate nature of the process.

JOCHMANN'S PROTEOLYTIC FERMENT AND THE UNKNOWN AGENT

The peculiar species and organ distribution of the pathogenic agent under investigation closely resembles the distribution of a proteolytic ferment studied by Jochmann.^{3,4,5} This ferment, Jochmann showed, is present in the bone marrow, spleen, and leucocytes—but not elsewhere—of man, monkeys, and, to a slight degree, dogs. It could not be found in any other species of animal examined. Jochmann considered that the ferment is peculiar to the leucocytes of certain species, and that in these species the occurrence of the ferment in spleen and bone marrow is due solely to the presence of leucocytes in these organs. I have undertaken experiments to determine whether the agent which is present in human bone marrow and spleen, and which is pathogenic for rabbits, has properties similar to those of the ferment described by Jochmann.

Jochmann found that his ferment is rapidly destroyed at 70° C., withstands 65° C. for one hour without appreciable diminution in activity, and persists indefinitely at temperatures of 55° C. and under. He, along with Lockemann,⁶ described a method by which the ferment can be extracted. They used bone marrow and spleen principally. Tissue is incubated for twenty-four to forty-eight hours at 55° C., during which time a considerable amount of autolysis takes place. Then five volumes of alcohol-ether (two volumes of absolute alcohol to one of ether) are added. After twenty-four hours at room temperature the supernatant fluid is removed and discarded; then the deposit is dried in a desiccator and afterwards mixed with an equal volume of 50 per cent. glycerin in water. After another twenty-four hours at room temperature the still undissolved deposit is removed and discarded, and the supernatant glycerin is mixed with five times its volume of alcohol-ether (proportion of alcohol and ether as before). This mixture is allowed to stand for twenty-four hours at room temperature to give time for the precipitation of material which has gone into solution in the glycerin. This final precipitate contains the ferment. The ferment is soluble in saline, and was generally used by Jochmann made up to twice the volume of the original tissue.

The question now was whether the agent which is present in human bone marrow and spleen, and which is pathogenic for rabbits, will withstand the severe treatment to which Jochmann submitted his leucocytic ferment. To obtain material for the necessary experiments human

bone marrow and spleen were extracted by the Jochmann-Lockemann method. In both cases the final saline solution was somewhat turbid at the time it was made. In the cold room a precipitate formed. This was spun down and removed. The clear brownish supernatant fluid was taken for use in the various tests to be described. Both the extract of bone marrow and the extract of spleen were shown to contain the leucocytic ferment, which, as already mentioned, is a proteolytic ferment. In the presence of either extract litmus milk gradually became clear owing to the action of the ferment on the casein.

Intracerebral and intrathecal injection of these first extracts which I had prepared by Jochmann and Lockemann's method produced symptoms resembling in all respects those which follow similar injections of saline suspensions of fresh tissue of human bone marrow and spleen. In most of the rabbits employed in these tests there was an incubation period of four days, during which the animals remained in apparently perfect health: in only one case was the incubation period as short as twenty-four hours.

A lytic action on brain constituents seemed possible, and several *in vitro* tests were made, all with the same result. Only one will be described.

The brain of an adult 2,000-gram rabbit was ground in 10 c.cm. saline in order to obtain a very thick suspension. Two similar tubes were taken: into one was placed 4.5 c.cm. brain suspension and 0.5 c.cm. saline, and into the other 4.5 c.cm. brain suspension and 0.5 c.cm. of Jochmann extract of human bone marrow. Both tubes were incubated at 55° C. After twenty-four hours the mixture in the tube containing emulsion and saline was found to be coagulated; it would no longer flow when the tube was inverted. In contrast to this the mixture of brain suspension and Jochmann-Lockemann extract had become more fluid in the twenty-four hours, and this process of liquefaction progressed steadily during the remaining days of the experiment. When comparative tests were made the extract of bone marrow proved to be a rather more powerful lytic agent than the extract of spleen. It is unlikely that the lysis of brain tissue just described can have been due to the presence of a lecithinase in the extracts. I made a number of experiments, but was unable to find such a ferment. An ovo-lecithin was used in the tests, and the proportions of extract and substrate were varied over a considerable range. Red blood corpuscles of the sheep were used as a test for split products due to the action of lecithinase.

Various modifications of Jochmann and Lockemann's method of extraction have been tried. The pathogenic agent, as also the proteolytic ferment—if that is a different thing—was found to be insoluble both in acetone and in methyl alcohol. Sometimes tissue for extraction has been treated with one and then the other of these two reagents between the period of autolysis and the first treatment with alcohol-ether. There is no obvious advantage in this, and, in fact, too prolonged an exposure to either methyl alcohol or acetone may, I suspect, result in a final product of somewhat reduced potency.

These modifications of Jochmann and Lockemann's method are mentioned chiefly as a further indication of the stability of the agent under investigation. It was found, too, that autolysis of the tissue is not necessary; this means that Jochmann and Lockemann's preliminary incubation can be omitted. Perhaps the simplest method of extraction which I have used is the following. The tissue—bone marrow or spleen—is extracted with acetone. At the end of fifteen minutes the undissolved material is collected on filter paper in a Buchner funnel, and washed first with absolute alcohol and then with ether. The resulting dry powder is then mixed with an equal volume of 25 or 50 per cent. glycerin in saline. At the end of twenty-four hours the undissolved material is spun out and the supernatant glycerin is mixed with five times its volume of alcohol-ether (two volumes of absolute alcohol to one volume of ether). The precipitate is removed and then taken up in saline.

To test whether the pathogenic agent is present in human leucocytes the following experiment was carried

out. Some 250 c.cm. of my own blood was run into a flask containing citrate solution. The blood cells were washed several times with citrate in the ordinary way, and then with saline. Finally, the leucocyte layer was removed and suspended in a very small volume of saline. Many red cells were still present, but this dilution was of secondary importance, since the cellular suspension obviously contained a greater concentration of leucocytes than had been present in the suspension of bone marrow employed for the intrathecal and intracerebral injections. Two rabbits, each of about 2,000 grams in weight, received intrathecally 0.4 c.cm. of the leucocyte suspension. Characteristic and fatal encephalitic symptoms developed in one animal, and showed that the pathogenic agent is present in human leucocytes just as the leucocytic ferment of Jochmann is.

If it could be shown that the pathogenicity of Jochmann-Lockemann extracts of human bone marrow, spleen, and leucocytes runs parallel with the ferment activity, then the identity of the pathogenic agent might be considered as established. This method of investigation has not been attempted. My experience is that quantitatively accurate comparisons are very difficult to obtain by the method of intrathecal and intracerebral injection in rabbits.

DISCUSSION

The conclusions drawn from the experiments which have been described above may now be briefly stated. First, the conclusion is drawn that the agent which is present in human bone marrow, spleen, and leucocytes, and which is pathogenic to rabbits, cannot be a virus. I find it impossible to believe that a living thing could survive the drastic treatment demanded by the Jochmann-Lockemann method of extraction. Secondly come conclusions as to the relationship between the pathogenic agent and the ferment. Here somewhat greater caution is necessary, but it has to be admitted that identity is suggested very strongly by the similarity in species and organ distribution of the two, by the similarity in resistance to heat, and by similar stability in the presence of alcohol, ether, alcohol-ether, methyl alcohol, and acetone.

But although I am satisfied that the pathogenic agent found in human spleen, bone marrow, and leucocytes cannot be a living virus, the whole phenomenon still remains a puzzle. The effects of intrathecal and intracerebral injections in rabbits differ from the known effects of chemical substances—except those of toxins like diphtheria and tetanus—in that they are progressive in character. In this progressive character, following an incubation period, there is a close resemblance to infectious diseases. Thus in a prolonged case the clinical course may run much as follows. After an incubation period of about four days a progressive rigidity of the hind legs sets in. A week or so later these symptoms subside, and the animal gradually returns to a more or less normal condition, which may persist for a week or two. Then the hind legs become paralysed, and a week or two later the fore legs also become paralysed. Eventually the animal dies of a general paralysis.

Lack of material has prevented me from testing whether the agent which Gordon discovered in lymphadenoma glands can be extracted by Jochmann and Lockemann's method, but a prolonged experience with the pathogenic agent in bone marrow permits a closer comparison of the clinical manifestations of the two than was possible at the time of publication of the paper by Friedemann and Elkeles in 1933. It is now clear, for instance, that provided the route of administration is the same, there is no demonstrable difference between the clinical effects produced by the agent from bone marrow and those produced by the agent from lymphadenoma glands. Gordon

described a rigidity of the legs as characteristic of his rabbits. He used intracerebral injections. Exactly the same thing is observed in rabbits which receive bone marrow intracerebrally. When, however, bone marrow suspension is given intrathecally, as in our original experiments, flaccid paralysis is the characteristic feature, though occasionally rigidity is seen. When bone marrow is injected intracerebrally, then rigidity is the characteristic consequence, just as in Gordon's rabbits. It is all a matter of technique. We have found, too, that, when tissue emulsions have been heated for one hour at 65° C. and then injected, it matters little whether the intracerebral or the intrathecal route is used; rigidity of the legs is a common feature in either case. Further, Gordon reported that his agent is remarkably stable in the cold. I find that the stability of the bone-marrow agent varies with the conditions of the experiment.

If bone marrow be ground in saline and then left in the cold room (4° C.) it loses its pathogenic activity rapidly—often within an hour. Whether there is an actual destruction of the pathogenic agent under these conditions has not been determined. The observed facts are that, when bone marrow is rubbed up in saline as described, a coagulation of the fatty matter soon sets in, clumps are formed, and the presence of the agent can no longer be demonstrated. Probably the agent is gathered up in the clumps. With alteration in technique the results alter. In the presence of 5 per cent. glycerin in saline the clumping sets in very slowly and the clumps are soft and easily broken down again. If the soft coagulated material be rubbed up in a mortar the fatty matter may be removed without difficulty. Glycerinated material has now been kept for six days in the cold room, rubbed up in a mortar, spun free of cells and gross particulate matter, and has then proved pathogenic for rabbits. Both agents are alike, too, in that they withstand a temperature of 65° C. Thus we reach the conclusion that the agent found in normal human bone marrow resembles the agent found in lymphadenoma glands in every material particular. Identity cannot, however, be said to be completely and finally established as yet.

CONCLUSIONS

Studies of the agent which is present in normal human bone marrow, and which Friedemann and Elkeles found to be pathogenic for rabbits, have been continued.

It is shown that this agent cannot be a living virus, since it withstands treatment which no living virus could be expected to withstand.

It has now been demonstrated that the agent, in addition to being present in human bone marrow and spleen, is present in human leucocytes.

The peculiar species and organ distribution which is displayed by this agent has been shown to be paralleled by the distribution of the proteolytic ferment which Jochmann found in normal human leucocytes. Whether the agent and the ferment are really identical remains to be finally established.

Increased familiarity with the pathogenic agent which we have studied still reveals no material difference between it and the agent which Gordon discovered in glands taken from cases of human lymphadenoma.

My indebtedness to Dr. Gordon has been mentioned already. It is now my pleasure to acknowledge financial assistance from the Rockefeller Foundation and the hospitality extended to me by the Medical Research Council at their Mill Hill laboratories.

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SOME PROPERTIES OF THE ENCEPHALITOGENIC AGENT IN LYMPHADENOMATOUS TISSUE

WITH FURTHER OBSERVATIONS ON GORDON'S BIOLOGICAL TEST IN THE DIAGNOSIS OF HODGKIN'S DISEASE*

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Much interest has been aroused in the aetiology of Hodgkin's disease by the work of Gordon (1933), who found that rabbits injected intracerebrally with lymphadenomatous tissue frequently developed highly characteristic nervous lesions. This reaction applied as a biological test has proved valuable in the differentiation of Hodgkin's disease from other forms of lymphatic hyperplasia (van Rooyen, 1933), but the exact nature of the pathogenic agent and its aetiological relationship to the disease is still obscure. An attempt has therefore been made to gain further information concerning its nature by a study of its behaviour towards certain physical and chemical agents, and the data obtained, together with further observations on the clinical value of the biological test, are incorporated in this paper.

Recently Friedemann and Elkeles (1933) have reported that the intracerebral inoculation of rabbits with bone marrow derived from cases of acute leukaemia and pernicious anaemia produces effects very similar to those described by Gordon. In May, 1933, the writer made a similar observation with bone marrow removed from a case of acute myelogenous leukaemia, and there was certainly a close resemblance between the experimental condition produced by this material and the syndrome resulting from the inoculation of lymphadenomatous tissue. The further question therefore arises as to whether any relationship exists between the pathogenic agent present in the bone marrow in the leucoses and that present in the lymphatic glands in Hodgkin's disease.

FURTHER RESULTS WITH THE BIOLOGICAL TEST

The following are a list of cases in various parts of the country which have been investigated by means of the biological test.

Biological Diagnostic Test (Gordon)

Case No.	Patient's Initial	Specimen Supplied by—	Institution	Histological Diagnosis	Result of Test
H9	T.	Dr. Chalmers Watson	Royal Infirmary, Edinburgh	Hodgkin's disease	Negative
H10	J.	Dr. Goodall	"	"	"
H12	R.	"	"	"	Positive
H14	F.	Dr. Matthews	"	"	"
H15	L.	Prof. Murray Lyon	"	"	Strongly positive
H18	H.	Prof. Murray Lyon	"	"	"
H26	H.	Prof. Fraser and Dr. Eason	"	"	Negative
H32	C.	Dr. Goodall	"	"	"
H17	C.	Dr. F.E. Reynolds	Stobhill Hospital, Glasgow	"	Positive
H19	B.	Dr. Cruikshank	Royal Infirmary, Glasgow	"	Negative
H21	S.	"	"	"	Positive
H23	McS.	"	"	"	"
H28	McL.	"	"	"	"
H33	H.	"	"	"	Strongly positive
H37	G.	"	"	"	Positive
H40	C.	Prof. Bramwell	Royal Infirmary, Edinburgh	"	Strongly positive

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† Previously published. See van Rooyen (1933).
‡ " " See Ogilvie and van Rooyen (1933).