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Effect of Nitrogen Mustard on Virus of Serum Hepatitis in Whole Blood.* (19607)

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The increasing use of blood and blood products in civilian and military medicine has brought to the fore, as one of the most urgent problems, the control of serum hepatitis (SH). The natural history of this virus disease is still obscure, but it has been definitely established that a carrier state may exist in individuals without a known history of jaundice and that such persons may harbor the virus in their blood in the absence of abnormal liver function tests(1). This situation obviously prevents the proper selection of safe donors. It is essential, therefore, to devise methods to sterilize blood and blood products. As far as plasma and sera are concerned an effective method appears to be at hand. Ultraviolet irradiation of these products has decreased the subsequent incidence of SH although the full extent of its efficacy has not as yet been critically evaluated. On the other hand, sterilization of whole blood has not been accomplished at present. In view of the relatively high incidence of the disease following transfusions the need for a satisfactory method of inactivating SH virus in whole blood is most urgent. The experience gained with nitrogen and sulfur mustards in the inactivation of various viruses seemed to offer an approach to this problem. It was first noted by Bawden and Pirie that mustards abolished the infectivity of certain plant viruses, the agents causing tobacco mosaic and bushy stunt disease, as cited by Gilman and Phillips(2). Later it was reported that sulfur mustard inactivated the viruses of rabies, Eastern equine encephalomyelitis, and hog cholera(3), and that both sulfur and nitrogen mustards rendered influenza virus non-infective(4). Herriott(5) studied the rates of inactivation of 6 animal, one plant, and 2 bacterial viruses by mustard gas and noted that those containing desoxyribose nucleic acid were more rapidly affected than the viruses containing ribose nucleic acid. Hartman *et al.*(6,7) demonstrated that the nitrogen mustard salt (methyl-bis- (beta-chloro-ethyl) amine hydrochloride) was an effective virucidal and bactericidal agent when added to plasma or whole blood in a concentration of 500 mg per liter. Plasma or blood thus treated, and subsequently neutral-

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ized with sodium thiosulfate, revealed only minimal toxicity for dogs and human beings. Studies on the components of blood indicated that the treatment induced some chemical changes which, however, in the opinion of these investigators, were not sufficiently severe to render the plasma or blood unsuitable for intravenous administration(8,10). Later it was noted that, under certain conditions, a toxic "protein-imine" (presumably formed by coupling of the dissociated chemical compound with plasma proteins) produced leucopenia in young mice when injected in large quantities(9). As a result, conditions of treatment to minimize this hazard have been recommended. The clinical use of nitrogen mustard-treated plasma has been reported by McClure *et al.*(10). No unfavorable reactions nor hematologic changes in adults or older children were observed among 400 patients given approximately 975 transfusions. It was reported that leucopenia developed in very young children transfused with the material but that these cases occurred prior to the recognition of the possibility of the formation of the toxic "protein-imine."

Hampil *et al.*(11), using a technic suggested by Mangun(12) for the sterilization of whole blood† found that a concentration of 0.5 mg nitrogen mustard per ml of whole blood artificially contaminated with high-titered Western or Eastern equine encephalomyelitis virus did not completely inactivate either virus. However, in view of its inactivating effect on other viruses studied under these conditions of exposure, this chemical appeared to offer a possibility of success for the inactivation of SH virus. Furthermore, relatively slight chemical changes occurred in the various components of whole blood under these circumstances(11).

In the present investigation an attempt was made, therefore, to sterilize whole blood obtained in the pre-icteric stage from volunteers infected with SH virus, by treatment with nitrogen mustard prior to intravenous injection into another group of volunteers. Under the conditions of the experiment active virus remained after the treatment.

Methods and materials. Virus. The SH virus used was obtained originally from a plasma pool collected at Fort Bragg. The virus had undergone 2 passages in volunteers. Sera of individual patients of the second group, drawn between the 40th post-inoculation day and up to the dates of first development of positive liver function tests, were pooled and several such pools were combined and used, after appropriate sterility and safety tests, for infection of the blood donors needed for the present experiment. The volunteers included in the present study were healthy, young males between the ages of 21 and 33 years. Each man had a physical examination, liver function tests, complete blood count, and urine analysis prior to acceptance for the experimental study. Following inoculation, they were seen daily by a physician or a nurse. From the 40th post-inoculation day until discharged from the project, urine analyses were performed daily and liver function tests once or twice weekly. The tests have been described previously(13). *The preparation and treatment of icterogenic whole blood.* Two ml of pooled pre-icteric serum were injected intramuscularly into each of 12 volunteers. Only one of these failed to show any signs of illness, 3 developed hepatitis without jaundice, and 8 became jaundiced. The average incubation period was 81 days with a range from 68 to 88 days. On the 69th day after inoculation, *i.e.*, at the time of the first clinical signs, blood was collected from 4 of the volunteers belonging to blood group Type O, RH negative. The bottles which were to be used for collecting the infected bloods were prepared the day prior to bleeding as follows: 25 ml of ACD solution pH 4.9 were pipetted into each of a series of bleeding bottles. To half of these (labeled "Nitrogen Mustard") 0.6 ml of 2.5 M NaOH was added to give a final pH of 6.2. The alkali was calculated to be sufficient to neutralize the acidity of the nitrogen mustard hydrochloride to be added and to produce a pH of 7 to 7.2 in the presence of whole blood. The other half of the bottles not receiving the alkali were labeled "control". A vacuum was produced in all bottles and they

† This technic, designed to suppress the formation of toxic substances, was identical with that employed in experiments on application of the compound to the SH blood described in this paper.

TABLE I. Results of Intravenous Injection of Nitrogen Mustard Treated Whole Blood and Non-Treated Blood into 10 Volunteers (Group B) in Each Test.

Treatment of blood	Dilution	Hepatitis with jaundice No.	%	Hepatitis without jaundice No.	%	Negative No.	%
HN_2	Undiluted	2	20	3	30	5	50
None	"	1	10	2	20	7	70
"	1–1000	3	30	4	40	3	30
"	1–1000000	2	20	3	30	5	50

were autoclaved for 15 minutes, after which the pressure was brought down rapidly. A total of 200 ml of blood was drawn from each of the donors, 100 ml each into a "control" and "nitrogen mustard" bottle, respectively, to yield a final volume of approximately 125 ml. The bottles marked "nitrogen mustard" were kept inverted, as in bleeding, and, immediately after drawing of the blood, 6.25 ml of a 1% solution of nitrogen mustard was injected into the bottle through the stopper. This provided a final concentration of approximately 0.5 mg per ml, or 500 mg of HN_2 per liter of blood. The bloods were swirled during the addition, thoroughly agitated thereafter to insure satisfactory mixing and placed in a 37°C waterbath for 5 minutes and at room temperature for 3½ hours, thereafter.

The solution of HN_2 was prepared as follows: Weighed amounts of dry nitrogen mustard were placed into 100 ml vaccine stoppered bottles. Immediately prior to use, sterile cold pyrogen-free distilled water was injected into the bottle in sufficient amounts to make a 1% solution. Two separate bottles were used, one containing 280.7 mg of the hydrochloride salt to which 28.1 ml of water were added and another bottle containing 337.5 mg of the chemical to which 33.8 ml of water were added. The solutions were kept in a cold water bath and were used for not more than 20 minutes after preparation. Hypodermic syringes were used for making all transfers. After the 3½ hours of incubation of the bloods with HN_2, 0.6 ml of a solution of 1 M sodium thiosulfate was added to each bottle. After thorough mixing the treated and control bloods were pooled separately under aseptic conditions. The pools were then stored at 4°C for 5 days prior to transfusion in an effort to simulate conditions of storage that prevail in most blood banks.

Results of intravenous injection of HN_2-treated and untreated blood. In an evaluation of the virucidal properties of a compound the concentration of the infectious agent used obviously may influence the results. An attempt was made therefore to obtain some information as to the quantity of virus present in the blood but in experiments requiring volunteers titrations are feasible only within limits. Consequently, the 40 available volunteers were divided into 4 groups of 10 each. To the first group of 10 were given 50 ml of the nitrogen mustard treated preparation intravenously; the second group of 10 received intravenously 50 ml of the untreated preparation, both representing 40 ml of whole blood. Groups 3 and 4 received 40 ml of 10^{-3} and 10^{-6} dilutions of untreated preparation, respectively, or 0.04 and 0.00004 ml of whole blood.

The results of this experiment are shown in Table I. It is seen that the nitrogen mustard as used was ineffectual in preventing serum hepatitis. There were 2 cases of hepatitis with jaundice and 3 without jaundice resulting from injection of the nitrogen mustard-treated blood as compared to one case with jaundice and 2 without jaundice in the control group. In the 2 groups given control blood diluted to 10^{-3} and 10^{-6} there were 3 and 2 cases of hepatitis with jaundice and 4 and 3 of hepatitis without jaundice, respectively. The average incubation period was 78.6 days with a range of 68 to 84 days. There were no toxic effects noted in the 10 men who received the nitrogen mustard-treated blood.

Discussion. On the basis of the inoculation of 50 ml of untreated blood diluted to 10^{-6} and a correction for the dilution factor introduced by the ACD solution 1 ml of the pooled blood contained at least 25,000 infectious doses of SH virus and possibly more. There

is no doubt that nitrogen mustard at a concentration of 500 mg per liter failed to render the blood safe for transfusion.

In evaluating this result several possible factors must be considered. 1. It may be that nitrogen mustard failed to combine with the protein of the SH virus and thus failed completely to inactivate. This is not compatible with the suggestive results obtained with icterogenic plasma treated with nitrogen mustard(7). 2. It is not known whether the virus may not be harbored within erythrocytes or white blood cells. If it is located inside cellular components of the blood it is possible that the concentration of nitrogen mustard within the respective cells was too low and thus the virus particles were not inactivated. 3. It is conceivable that the nitrogen mustard may have inactivated a large proportion of the virus particles but there remained enough in the active state to induce infection in the volunteers. As a matter of fact only 1 in 10^6 particles may have escaped inactivation in the experiment reported. This interpretation could have been verified only by titrating also the HN_2 treated blood. In any case, such highly infectious bloods presumably may be drawn by chance from blood donors in the late incubation period or possibly even from carriers of SH virus in the routine collection for blood banks. The donors of the infective blood in this study were bled 3-9 days prior to the onset of jaundice, in the assumption that the preicteric stage may represent the time at which maximal titers of virus are obtained in the blood. However, it is not known whether this might not occur earlier in the incubation period. It is likely that this experiment constituted a particularly severe test for the inactivating capacity of HN_2 and that possibly under routine conditions, where asymptomatic carriers and not incubatory carriers may be the prime source of infection, the HN_2 treatment may have given more encouraging results. However, it is obvious that the ideal sterilizing agent of whole blood should be effective against maximal possible concentrations of SH virus.

The result obtained with the technic of HN_2 treatment used was similar to those noted with some other viruses, notably the agents of Western and Eastern equine encephalomyelitis. These were not completely inactivated in artificially contaminated whole blood by HN_2, whereas other viruses became non-infective. Thus, it would seem that SH virus belongs to the more resistant viruses, which would be compatible with other information with regard to its stability. Higher concentrations of HN_2 and longer exposure of SH virus to this chemical might prove to be more effective but in that case the danger of toxic byproducts of the reaction and changes in the blood constituents may prevent the safe use of the blood for transfusions.

Summary. 1. Nitrogen mustard in a concentration of 500 mg per l failed to inactivate the Fort Bragg strain of serum hepatitis virus in whole blood. 2. Possible reasons for this failure are discussed. 3. The virus titer of the whole blood used was found to be at least 25000 infectious units/ml.

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