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Enhancement of Diphtheria Toxin Activity by Heat-Killed Bacteria and Red Blood-Cells*

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No generally accepted theory has been proposed so far to explain the malignant course of toxic diphtheria which still occurs rather frequently in countries with high epidemicity of this disease.

When ANDERSON and col. described in 1931/33 three different types of Corynebacterium diphtheriae, the given nomenclature "*gravis, intermedius, mitis*" implied that the three bacterial types caused, according to their names, infections of different severity, an opinion which has not been fully corroborated in the following years (FROBISHER, 1943; PESTANA et al., 1939). Another hypothesis had been put forward earlier by ROUX and YERSIN, in 1890, who explained the enhanced virulence of diphtheria bacilli in malignant cases by a mixed infection, especially with streptococci. In the same direction also lie the experiments by RAMON and DJOURICHITCH (1934) demonstrating a raised virulence of cultures of C. diphtheriae after addition of living or heat-killed streptococci, or even of pure broth or tapioca flour. These authors suggested that the "activating" substances modify in some way the local terrain which thus becomes more susceptible to the proliferation of diphtheria bacilli. Similar experiments by UPDYKE and FROBISHER (1947) in mice and rats gave an equivocal result.

This line of investigation has later been completely abandoned in spite of the striking analogy which exists between the enhancement of virulence of C. diphtheriae by mixed infections and the toxin activation ("potentation") produced by a great variety of organic substances. (RICKETTS and KIRK, 1906; BROFENBRENNER, 1923/24; DE WAELE). The various mechanisms which explain the activating effect of serum, lecithin and other biological materials on exotoxins (tetanus, diphtheria, botulinus, etc.) have been discussed in detail by SOMMER and SOMMER (1928), and by WRIGHT (1955).

Activated toxins, when assayed in animals, display an increased potency up to ten times higher and more than the pure toxin diluted in physiologic saline. In recent experiments (EICHBAUM [1], 1963), we have shown that the activation of diphtheria toxin by dead bacterial bodies or by red blood-cells not only causes a very significant enhancement of the lethal effect but, in addition, it also produces very severe organ lesions, hardly ever found after subcutaneous

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injections of pure[1] toxin. Such an activating effect is brought forth by substances which may be present also under natural conditions at the very place of bacterial invasion or implantation. This points to the potential influence of unspecific activating factors which might be operative in the pathogenesis of human diphtheria, especially its malignant form.

The present paper describes the special experimental conditions of diphtheria toxin activation and the characteristic histologic lesions produced by activated toxin, finally discussing the pathogenetic mechanisms involved.

Material and Methods

Guinea-pigs of either sex, with an average weight of 250 g were injected *subcutaneously* with sublethal doses of diphtheria toxin and/or one of the "adjuvants" to be described below. Each experimental series comprised 3 parallel lots of 10 animals, the first lot receiving diphtheria toxin alone, the second one the "adjuvant", the third one a mixture of toxin + "adjuvant".

Material injected

1. Diphtheria toxin. a) Commercial toxin n^0 1014 (Instituto Pinheiros, Produtos Farmacêuticos S.A., São Paulo) containing 350 MLD[2] per ml. b) Purified diphtheria toxin RX 7238 (The Wellcome Research Laboratories. Beckenham, England) containing 120.000 MLD per ml. All dilutions were prepared with sterile physiologic saline, immediately before injection. The toxin doses, which varied between $^1/_4$ to 1 MLD, were contained in a 0.5 ml volume.

2. "Adjuvants". Heat-killed bacteria: A test tube containing 5 ml of a 24 hour bacterial culture was centrifuged at 1500 r.p.m. during 2 minutes. The sediment, after three washings with physiologic saline, was resuspended in 5 ml of saline and put into a water bath at 100^{0}C, during three minutes. After cooling down, 0.5 ml of this suspension was mixed with 0.5 ml of diphtheria toxin ($^1/_2$ MLD) and injected immediately afterwards into the guinea-pig by subcutaneous route. The contact between bacteria and toxin, before injection, lasted not more than 5 minutes at most.

The following bacteria and substances were tested:

1. *Corynebacterium diphtheriae*, strain "Park 8", grown in Legroux-Ramon's medium during 24 hours at 37^{0}C. 2. *Staphylococcus aureus*, strain "Wood 46 CN 56 alpha", grown in Legroux-Ramon's medium during 24 hours at 37^0 C. 3) *Escherichia coli*, strain D642, grown in glucose broth, during 24 hours at 37^0 C. 4. *Streptococcus haemolyticus*: Lancefield group A, strain "Dochez Ly 5", grown in glucose broth during 24 hours at 37^0 C. 5. *Haemophilus pertussis*, strain 10536; after growth on Levinthals agar during 24—48 hours, the cultures were washed off and suspended in physiologic saline. 6. *BCG*, commercial oral vaccine (Instituto Butantã). 7. Active charcoal (Merck) 10 per cent suspension in physiologic saline. 8. *Sheep red blood-cells* were obtained from whole blood, which had been defibrinated by agitation with glass-beads. After centrifugation and three washing with physiological saline, 0.5 ml of the sediment were mixed with $^1/_2$ MLD of diphtheria toxin, also contained in a 0.5 ml volume.

In some experiments, the activating effect of hemolyzed red-blood-cells, supernatant and ghosts respectively, was tested. For this purpose 35 ml of destilled pyrogenfree water were added to 35 ml of washed blood-cells. The hemolyzed blood was centrifuged at 1500 r.p.m. for 15 minutes, the supernatant fluid ("hemoglobin solution") was drawn off and the almost invisible sediment ("ghosts") was resuspended in 12 ml saline. For testing the potentiating effect of both fractions, $^1/_2$ MLD of diphtheria toxin in a 0.5 ml volume was mixed with 1.0 ml of the hemoglobin solution or of the ghosts, respectively. 9. *Guinea-pig blood-cells*, obtained by cardiac puncture, were prepared like sheep blood-cells (item 8). In some experiments the blood was drawn from the animal's own heart and was quickly reinjected by subcutaneous way after mixing it with $^1/_2$ MLD of diphtheria toxin.

[1] The term "pure" toxin will be used in the following to designate a toxin diluted in physiologic saline without addition of any "activating" substance.

[2] Minimal lethal dose.

Results

I. Death Rates Produced by Activated Diphtheria Toxin

As shown in Table 1, $^1/_2$ MLD of pure diphtheria toxin kills 23 out of 125 guinea pigs (18%) within 15 days, following the injection. The admixture of various adjuvants such as heat-killed diphtheria bacilli, coli bacilli, sheep red blood-cells or even of blood-cells of the own species (guinea-pig) is followed by an apparent rise of the death rate. Most deaths occurred between the 5th and the 10th day after the injection of activated toxin. The injection of the adjuvant did not cause any deaths.

On statistical analysis (Table 1), the increase of death rate after admixture of an adjuvant proved significant only for the combination of diphtheria toxin + diphtheria bacilli and of diphtheria toxin + red blood-cells, whereas the activation through addition of heat-killed coli bacilli might also have occurred by chance. No activating effect was obtained by mixing heat-killed streptococci, BCG, H. pertussis or charcoal, respectively, with diphtheria toxin. The combination staphylococci + diphtheria toxin, though slightly reducing the death rate, nevertheless caused very severe histologic kidney and heart lesions (see below).

Table 1. *Death rates produced by subcutaneous injection of diphtheria toxin and by diphtheria toxin activated with heat-killed bacteria and with red blood-cells*

	Substance injected	Number of deaths* / Total number of injected guinea-pigs	Death rate per cent
A	Diphtheria toxin**	23/125	18
B	Diphtheria toxin** + Diphtheria bacteria***	30/95 $P<0.05$	32
C	Diphtheria toxin** + Coli bacteria***	15/60 not significant	25
D	Diphtheria toxin** + Staphylococci***	4/30 not significant	13
E	Diphtheria toxin** + Sheep red blood-cells	41/85 $P<0.01$	48
F	Diphtheria toxin + Guinea-pig red blood-cells	9/20 $P=0.01$	45

* 1 Death occurring within 15 days after injection.
** $^1/_2$ MLD.
*** Heat-killed.

Since red blood-cells proved to be the most potent activator of all, this "adjuvant" was used for studying the phenomenon of toxin potentiation under varying conditions: identical results were obtained, whether the mixture toxin + adjuvant was injected after a contact time of 5 or of 120 min, at room temperature. If adjuvant and toxin were applied at the same time but at different places, e.g. abdominal and dorsal region, no activation occurred. It also failed to appear, when the toxin injection preceded the blood injection for 24 hours, though both had been injected at the same site. If however the order was reversed, i.e. if the blood-cell injection was followed after 24 hours by the toxin application,

both injections being applied at the same place, a very pronounced activation took place. The lethality in this case amounted to 18 deaths in 30 animals (Death rate = 60 per cent).

When a red blood-cell suspension was dissociated into hemoglobin solution and ghosts, by addition of destilled water, only the Hb solution retained a marked potentiating effect (death rate = 50 per cent). [Table 2].

Table 2. *Death rates produced by diphtheria toxin and by diphtheria toxin activated with red blood-cells, hemoglobin and ghosts*

	Substance injected	Number of deaths* / Total number of injected guinea-pigs	Death-rate per cent
A	Diphtheria toxin**	10/40	25
B	Diphtheria toxin** + sheep red blood-cells	23/40 $P<0.01$	57.5
C	Diphtheria toxin** + hemoglobin solution (supernatant)	20/40 $P<0.05$	50
D	Diphtheria toxin** + ghosts (sediment)	15/40 not significant	37.5

* Death occurring within 15 days, after injection.
** $^1/_2$ MLD.

In view of these findings we advanced the hypothesis that the interaction between diphtheria toxin and "activator" might give rise to the formation of a secondary, toxic substance, in vivo, which might be responsible for the enhanced lethality and the severe histologic organ lesions. We therefore tried to discover in the blood of these animals an active principle that could be transferred passively unto other, normal guinea-pigs or which might give a serologic reaction in vitro.

Thirty guinea pigs were injected subcutaneously with a mixture of sheep red blood-cells and diphtheria toxin ($^1/_2$ MLD).

The animals were divided into two groups of 10 and 20 guinea-pigs, respectively. In the first group with 7 survivors out of 10 injected animals, blood was obtained by cardiac puncture on the 5th day after injection and, in the second group with 16 survivors out of 20 injected animals, on the 7th day after injection. The blood samples of the individual animals, in the 5 days group and in the 7 days group respectively, were pooled and after clotting the sera were separated. Part of these sera was set apart for serologic testing, part of it was used for the injection of 14 normal guinea-pigs which received each 3 ml of serum intravenously.

All these animals survived. None of them, when sacrificed 30 days later, showed any lesions of internal organs.

In order to discover the presence of precipitating antibodies in these sera, Ouchterlony't agar-diffusion method was used. Both pooled sera were tested in several parallel sets againss pure diphtheria toxin and against the following antigens which had been homogenized in a high-speed rotating blender: suspension of heat-killed diphtheria bacilli, guinea-pig heart, kidney, liver, adrenal gland.

No precipitation bands were noted in any case.

It finally seemed of interest to find out, whether the MLD values became altered in a diphtheria toxin assay by Ehrlich's method, using a blood cell suspension

as diluting menstruum instead of physiologic saline. Taking the 96th hour after inoculation as the end point of the experiment, there was no difference in the MLD value of toxins diluted with either red blood-cell suspension or physiologic saline. This observation is at variance with the mentioned potentiation of tetanus, botulinus and diphtheria toxin, where the admixture of lecithin, serum or other substances produced a considerable rise of the toxin titer.

II. Histologic Lesions Produced by Activated Diphtheria Toxin

The most constant and severest lesions after injection of activated diphtheria toxin occurred in the *kidney*. They appeared with the same frequency in animals that had been killed by the mixture of bacteria + toxin, as in those killed by blood-cells + toxin, though in the latter case the death rate was much higher (Table 1). The combination red blood-cells + toxin seemed, however, less

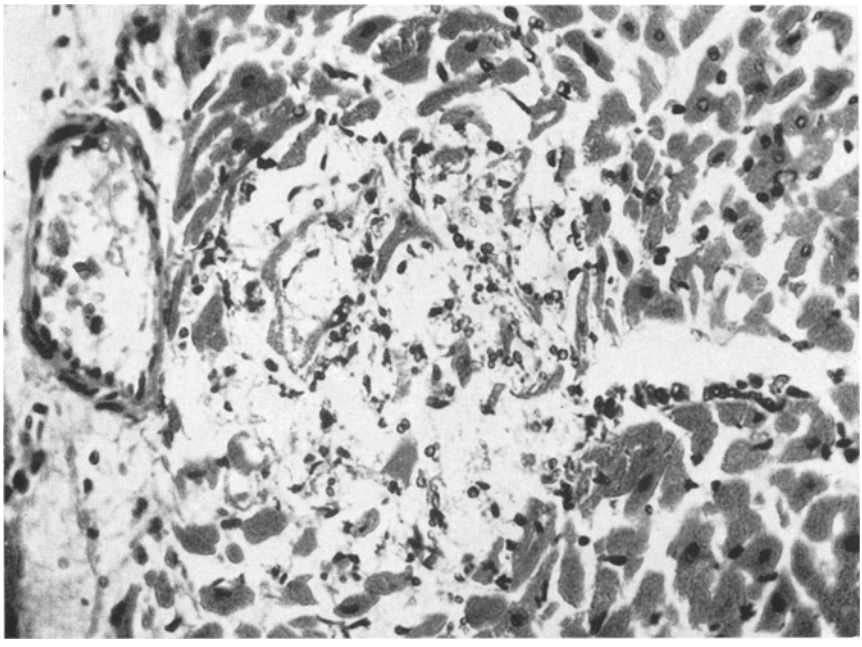

Fig. 1. Diphtheria toxin $^1/_2$ MLD + heat-killed diphtheria bacteria: Death 5 days after subcutaneous inoculation (No. 56/68). Heart: Necrotic histolytic focus with cell debris. Surrounding myocardium without inflammatory reaction. Hematoxylin-Eosin × 250

effective in bringing forth degenerative *heart* lesions, which were seen more regularly after injection of diphtheria toxin + heat-killed bacteria (C. diphtheriae, E. coli, staphylococci). Except for the skin scars which developed at the site of subcutaneous injection of toxin pure or activated, no histologic lesions were found in animals surviving till the 30th day after inoculation. There was no significant difference in the size of necrotic skin lesion caused by either pure or activated toxin. The subcutaneous injection of the adjuvant alone (red blood-cells, heat-killed bacteria etc.) produced in no case any histologic lesions of internal organs.

Heart. No gross histologic alterations were found in guinea-pigs which succumbed to an overwhelming dose of *pure* toxin, within a 12 to 24 hours period.

In animals surviving for longer periods (2—6 days), there was fatty degeneration of myocardial cells, interstitial edema and focal accumulation of round cells and histiocytes. As we have pointed out elsewhere [EICHBAUM (1), 1963], such lesions are only rarely produced by a single injection of pure diphtheria toxin and are generally restricted to small areas of the heart muscle. On the other hand, severe heart lesions are frequently encountered in animals which had been killed by injections of activated diphtheria toxin. In the most violent cases, large areas

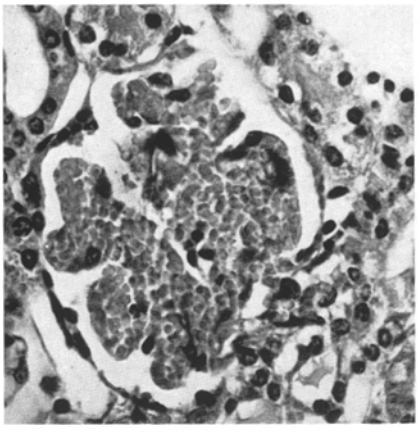

Fig. 2

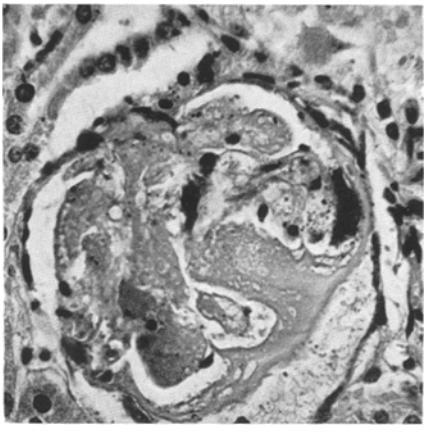

Fig. 3

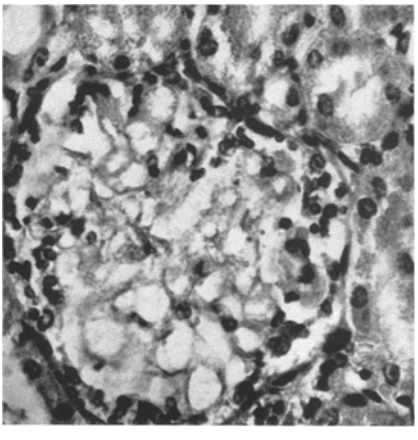

Fig. 4

Fig. 2. Diphtheria toxin $^1/_2$ MLD + heat-killed diphtheria bacteria: Death 5 days after subcutaneous inoculation (No. 56/68). Kidney: Hyperemic intraglomerular stasis with almost complete loss of epithelial and endothelial cells (Stage I). Hematoxylin-Eosin × 400

Fig. 3. Diphtheria toxin $^1/_2$ MLD + heat-killed E. Coli: Death 7 days after subcutaneous inoculation (No. 72/450). Kidney: Fibrinous degeneration of the glomerulus. Foamy exsudate within capsular space (Stage II). Hematoxylin-Eosin × 400

Fig. 4. Diphtheria toxin $^1/_2$ MLD + heat-killed diphtheria bacteria: Death 5 days after subcutaneous inoculation (No. 56/58). Kidney: Complete destruction of glomerulus; at its circumference some lymphocytes and cell debris (Stage III). Hematoxylin-Eosin × 400

of the heart musculature become honey-combed by numerous large holes of necrotic tissue (liquefaction necrosis) without any inflammatory reaction in the vicinity (Fig. 1). In other parts of these hearts usually appears an interstitial edema with accumulation of histiocytes, lymphocytes and isolated polymorphonuclear cells. In the surviving animals of this group, however, no histologic heart lesions were observed excepting a few cases where a small area of spongy scar tissue had remained as a residue of a previous focal necrosis.

The histological lesions of *lung* [EICHBAUM (2), 1963], *liver* and *spleen*, produced by activated toxin, do not differ from those caused by pure toxin or by diphtheria bacilli. Only exceptionally one meets larger foci of liquefaction necrosis

in the liver and in the spleen after injection of a mixture of heat-killed diphtheria bacilli + diphtheria toxin.

It is in the *kidney* where the most pronounced lesions are encountered, which are rarely missing in any animal killed by diphtheria toxin in its pure or its activated form. In animals killed by pure toxin, one generally finds only slighter renal lesions such as anaemic glomerular loops with partial loss of endothelial and epithelial cells and cloudy swelling of convoluted tubuli. More pronounced kidney lesions (glomerulo-necrosis) were found only in three of these cases out of a total of 150 examined. On the other side, very severe kidney lesions occurred in almost all animals killed by activated toxin, though the degree of tissue injury

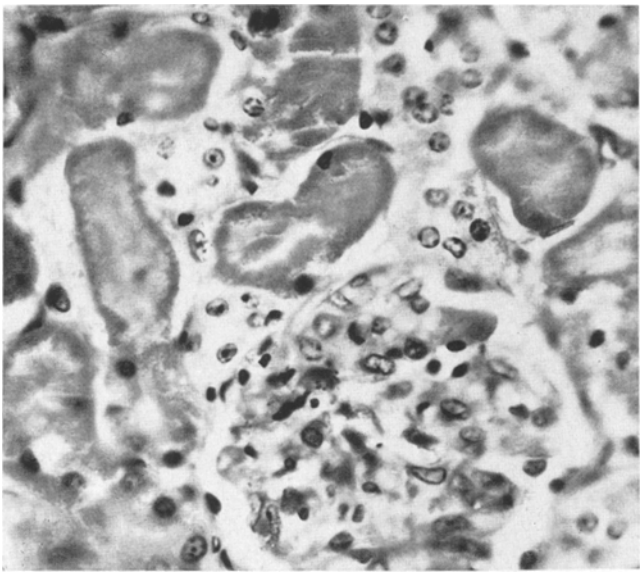

Fig. 5. Diphtheria toxin $^1/_8$ MLD + washed guinea-pig blood-cells Death, 7 days after subcutaneous inoculation (No 105/503). Kidney: Glomerulus comparatively well preserved. Severe degeneration of tubuli, which display an almost complete loss of stainable nuclei. Hematoxylin-Eosin × 400

varied greatly for each individual case. The glomerulus seems to be the primarily affected part. The degenerative process which finally leads to a complete glomerulo-necrosis, apparently passes through three successive stages which might sometimes coexist in neighbouring parts of the organ. The *first stage* is an intense intraglomerular hyperemia with partial or total loss of epithelial and endothelial cells. The *second stage* is a partial or complete fusion of red blood-cells (sludging) within the glomerular loops. A homogeneous or spiderweb-like exsudate forms within the capsular space. There is a thickening of the basal membrane. The swollen, partly fused glomerular loops and the intracapsular exsudate take a lively cherry-red colour with PAS stain and a deep blue colour with Weigert's Fibrin stain. In the *third stage*, which rarely comes to its full development, the glomerulus is completely destroyed displaying only a skeletal outline of its previous structure with a few isolated endothelial cells left (Figs. 2—4).

Strangely enough, it often happens that these severe lesions are restricted exclusively to the glomerulus with no or only slight degenerative changes of the

tubular apparatus. In other cases, the tubuli might show varying degrees of degeneration and necrobiosis which sometimes extend into the medulla. The most pronounced lesions are generally seen in the subcapsular cortical tubuli. In the severest cases, there is a complete destruction of the epithelial lining with vacuolisation or granular degeneration of protoplasma, loss of stainable nuclei and presence of homogeneous casts in the lumen. It only exceptionally happened that tubular changes were the sole lesions encountered, while any severe glomerular lesions were missing (Fig. 5). Hyperemia of interstitial blood vessels in the cortex was generally present and also, to a lesser degree, in the medulla. No intravascular thrombi have been noted anywhere.

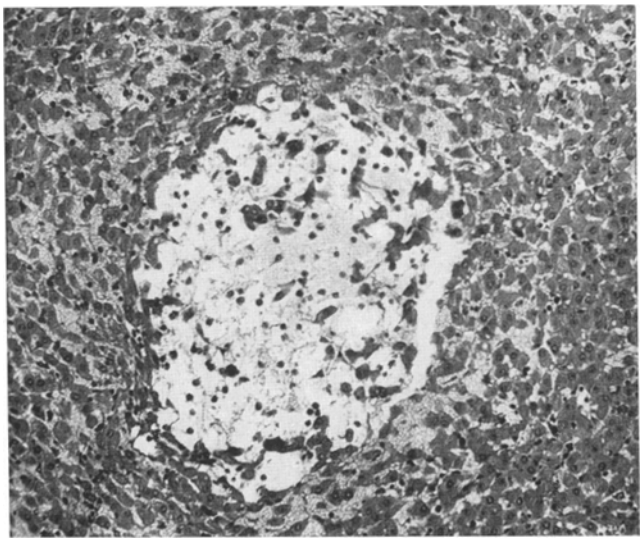

Fig. 6. Diphtheria toxin $^1/_2$ MLD + heat-killed diphtheria bacilli: Death 14 days after subcutaneous inoculation (No. 70/694). Suprarenal cortex: Focus of histolytic necrosis. No inflammatory reaction in the surrounding tissue. Hematoxylin-Eosin × 100

Suprarenals. Hemorrhagic necrosis which characterize the typical diphtheria intoxication were only rarely found after injection of activated toxin. There was instead, in most cases, an intense interstitial edema with large areas of focal non-hemorrhagic necrosis, mainly in the cortex. These necrotic areas sometimes appeared like punched-out holes within an otherwise normal tissue (Fig. 6).

Discussion

In the heart, activated toxins either provoke a focal interstitial myocarditis and/or severe degenerative-necrotic lesions of myocytes without major inflammatory reaction in the vicinity. The kidneys present as primary lesion a severe degeneration of the glomeruli, a process which sometimes seems to arise so quickly as to leave no time for the development of any morphologic alterations of the tubuli. When both parts, glomeruli and tubuli, are involved, the histological picture greatly resembles the general Shwartzman reaction (G.S.R.). As in the G.S.R., the surviving animals don't show any organ lesions: either the kidneys are affected and the animals die or they survive with intact organs. There are

several points, however, in the pathogenesis of the described phenomenom which separate it clearly from the classical G.S.R.: 1. endotoxin does not play any known rôle in the development of kidney lesions resulting from the injection of activated diphtheria toxin. 2. All injections are applied by subcutaneous route. 3. The phenomenon is observed in the guinea-pig, which generally passes for a species resistant to the production of the G.S.R. Our findings also resemble the renal lesions observed by FABER (1917) after intravenous injections of Coli bacilli or of "Vaughan's split protein" into rabbits which had received, several days before, small doses of diphtheria toxin, also by intravenous route. This association of diphtheria toxin + bacteria differs from our own experiments on toxin activation in so far, as in FABER'S experiments the injections were applied by intravenous route into rabbits, in an interval of 1 to 3 days between both injections. Similar degenerative kidney lesions can be obtained occasionally also by a single *intravenous* injection of pure diphtheria toxin into guinea-pigs [EICHBAUM (3)].

Two hypothesis are proposed which might explain the potentiating effect of adjuvants on diphtheria toxin. Diphtheria toxin, though devoid of an immediate enzymatic activity, causes within hours an intense local inflammation, which is followed by a skin necrosis at the site of injection. During the development of this inflammatory reaction, certain tissue enzymes are set free which might attack and decompose the injected adjuvant. Secondary split products thus liberated might add their toxic action to that of the injected diphtheria toxin.

One might also think that the adjuvant competes with some toxin-binding substances or that it facilitates, by some other unknown mechanism, the entrance of the toxin into the general circulation. This explanation is suggested by the fact that pure diphtheria toxin, when injected intravenously, sometimes causes kidney lesions quite similar to those found after subcutaneous injection of activated toxin.

A third hypothesis has apparently been disproved by our experiments, namely that the heart and kidney lesions might be produced by organ-autoantibodies whose formation could have been elicited by the double attack toxin + adjuvant. This was suggested by the observation that death rarely occurred before the 6th or 7th day after inoculation, i.e., only at a time when greater amounts of circulating antibodies would be expected. The presence of such antibodies however could not be demonstrated, neither by passive transfer to other animals nor by serological methods.

It must remain so far a matter of conjecture, whether similar activating factors as those here described might play any rôle in the pathogenesis and evolution of human diphtheria especially its malignant form. The experiments here reported prove at least, that the pathogenic activity of diphtheria bacilli is not completely defined by the strength of toxin, expressed in MLD. Numerous local factors existing at the primary site of diphtheria infection might greatly modify the toxin effect on internal organs.

Summary

The lethal effect of small doses of diphtheria toxin, injected subcutaneously into guinea-pigs, is greatly enhanced by the addition of certain adjuvants, such

as heat-killed diphtheria bacilli, coli bacilli or red-blood cells. Toxin activation takes place only when toxin and adjuvant are injected at the same site simultaneously, or when the injection of the adjuvant preceeds the toxin injection for 24 hours.

The activated toxin also causes much severer degenerative lesions in heart and kidney than pure toxin. Similar though less pronounced heart and kidney changes are found occasionally, when the pure toxin is applied by intravenous route. Identical degenerative processes have also been observed in human malignant diphtheria.

Die Verstärkung der Wirkung von Diphtherie-Toxin durch hitzegetötete Bakterien und Erythrozyten

Zusammenfassung

Die letale Wirkung kleiner Diphtherietoxinmengen, bei subcutaner Injektion in Meerschweinchen, wird durch Beimengung bestimmter aktivierender Substanzen („Adjuvantien" oder „Aktivatoren") wesentlich gesteigert. Als wirksamste Adjuvantien erwiesen sich rote Blutkörperchen, sowie hitze-getötete Diphtherie- und Colibacterien. Eine Aktivierung erfolgte nur, wenn Adjuvans und Toxin an derselben Stelle injiziert wurden, sei es gleichzeitig oder nacheinander in einem 24stündigen Intervall. Im letzteren Falle trat die Aktivierung nur dann ein, wenn die Injektion des Aktivators vor der Toxininjektion erfolgte.

Aktiviertes Toxin verursacht schwerste Herz- und Nierenveränderungen. Ähnliche, wenn auch an Intensität geringere Organschädigungen finden sich gelegentlich nach *intravenöser* Injektion eines reinen Diphtherietoxins. Auch bei klinischen Fällen maligner Diphtherie wurden analoge histologische Läsionen, vor allem in der Niere, beschrieben.

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