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A STUDY OF BASOPHILIC INCLUSION BODIES PRODUCED BY CHEMOTHERAPEUTIC AGENTS IN TRYPANOSOMES

BY

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In the previous paper a description was given of inclusion bodies produced in trypanosomes by antrycide. In this paper a more detailed study of the chemical behaviour of these inclusion bodies and a discussion of their significance will be given in three parts.

I. Methods by which inclusion bodies can be produced in trypanosomes and other types of cell, and their relation to basophilic granules that occur naturally in the cytoplasm of trypanosomes.

II. A study of the nature of these inclusion bodies by cytochemical techniques.

III. A mode of action for certain " trypanocidal " drugs.

I. METHODS BY WHICH INCLUSION BODIES CAN BE PRODUCED

(a) By trypanocidal drugs

Of the drugs that have so far been examined *in vivo*, antrycide, dimidium bromide, and suramin produced well-marked inclusion bodies, tryparsamide and stilbamidine did not produce basophilic inclusion bodies, acriflavine (a mixture of proflavine and its N-methyl derivative) caused the trypanosomes to degenerate so that although inclusion bodies could be detected they were not well formed and may have been altogether different structures.

Only two references to inclusion bodies produced in trypanosomes by chemotherapeutic agents have been found in the literature. Saito (1928) described granules in trypanosomes after treatment (presumably *in vivo*) with acriflavine, and these probably correspond to the ill-defined basophilic inclusion bodies produced by this drug which were referred to above. Lavier (1928) described " Paravacuolar formations " which were produced " in certain crises (unspecified) resulting in a cure for the host animal," and he distinguished them by vital staining from the naturally occurring " Volutin," " Chromatoid," or " Metachromatic " granules. Lavier's work is difficult to evaluate both for lack of experimental details and because the vital stains that he used to distinguish these two types of formation—pyronin, thionin, and methylene blue—can themselves create artifacts with properties similar to those that he was investigating, as will be shown later in this paper.

(b) By vital stains and other non-trypanocidal basic substances

An experiment designed to investigate the staining properties of these inclusion bodies in the living trypanosome was performed as follows: Trypanosomes showing inclusion bodies from an animal treated with antrycide were examined under the microscope in the wet smear with oil immersion objective, and a saturated solution of dye was added under the coverslip. Various dyes were used; those containing primary, secondary, or tertiary amine groups stained the inclusion bodies, and, of these, neutral red and toluidine blue were the most effective. No acid or quaternary amine dye was found which stained living trypanosomes. Under these conditions the inclusion bodies were the only structures in the trypanosome which were stained.

Normal trypanosomes were also stained by this technique and produced a most striking reaction. The dye was at first distributed evenly in the cytoplasm; then after about a minute (depending on the final concentration of dye in the diluting medium) the coloured cytoplasm gradually formed stained aggregates which were identical in appearance and distribution with the stained inclusion bodies produced in trypanosomes from an antrycide treated animal. The production of inclusion bodies in this way by neutral red (a concentration of 1/1,000,000 dye in Yorke's medium produced them in one hour) was found to be reversible, that is to say, if the trypanosomes containing these coloured granules were centrifuged free of neutral red containing solution, and resuspended in fresh diluting fluid, their appearance became normal when viewed both by ordinary and by phase contrast microscopy. When 40 mg. neutral red per 20 g. was given by intravenous injection to a mouse heavily infected with trypanosomes, and blood taken five minutes later and examined in the wet unstained smear, inclusion bodies could be seen with the same appearance and distribution as those produced by neutral red *in vitro*. After 30 minutes only a few of these inclusion bodies could be seen, and after 60 minutes they had all disappeared. In dry fixed smears inclusion bodies produced in this way could be stained with eosin-methylene blue compound stains; their staining properties were similar to those of the inclusion bodies produced by antrycide, except that the violet colour which they assumed was slightly less intense.

The formation of basophilic granules by basic substances in cells other than trypanosomes *in vitro* and *in vivo* is a phenomenon that has been studied intensively by other workers and has been reviewed by P. Dustin (1947). The studies have been made chiefly on nucleated red cells and reticulocytes but are applicable to many other cell types, the only requisite for the production of basophilic granules being a basophilic cytoplasm. Dustin identified this basophilic substance in nucleated red cells as ribonucleo-protein and the chief component of the basophilic granules as ribonucleic acid.

(c) Naturally occurring inclusion bodies or "Volutin" granules

L. van den Berghe (1942) investigated "Volutin" granules in a strain of *T. gambiense* which produced large numbers with great regularity and identified their chief component as ribonucleic acid, but as his only method of identification involved the use of non-crystalline ribonuclease his conclusion must be accepted with reserve. I have not been able to verify his findings for lack of a strain of trypanosomes that produces "Volutin" granules with regularity, but can find no

evidence to suggest that in their chemical nature the "Volutin" granules differ from inclusion bodies produced by drugs or by vital stains. It is probable that the "Volutin" granules (which occur only with certain strains of trypanosomes and in certain species of host) are an unspecific reaction of the trypanosome to non-trypanocidal basic substances circulating in the blood.

II. A CYTOCHEMICAL STUDY OF INCLUSION BODIES PRODUCED BY ANTRYCIDE

Mice infected with *T. equiperdum* showing 1 to 10 trypanosomes per oil immersion field were injected with antrycide methylsulphate (0.02 mg./20 g.), and smears were taken on glass and quartz slides when inclusion bodies had appeared in the cytoplasm of the trypanosomes. At 24 hours the inclusion bodies were fully developed and the trypanosomes otherwise normal. At 48 hours the inclusion bodies were the same, but the trypanosomes as a whole showed signs of degeneration.

The smears were fixed unless otherwise stated by heating at 160° C. for 20 seconds and by immersion when cool for two minutes in methanol.

Staining with Unna's carbol-pyronin-methyl green.—Slides (fixed by immersion for two minutes in methanol) were placed for twenty minutes in this solution at 37° C. The trypanosome nuclei stained a faint green and the inclusion bodies a full orange red.

Kurnick (1950) has shown that the polymerized state of desoxyribonucleic acid causes it to stain green specifically with methyl green. The orange red staining of ribonucleic acid with pyronin is not so specific, but in the presence of an intact nucleus staining with methyl green it indicates that the inclusion bodies are composed of cytoplasmic material probably containing ribonucleic acid.

Staining with Giemsa after treatment with specific enzymes.—As a general indication of the chemical composition of cells, a basophilic reaction to eosin-methylene blue compound stains shows the presence of nucleic acid, and an eosinophilic reaction shows the presence of protein (Pollister, 1950). More specific information can be gained by enzyme digestion of smears and subsequent staining with Giemsa, which is in this instance the most satisfactory variety of this general type of stain.

Incubation was performed at 30° C. in twice distilled water containing the enzyme, and a control preparation was treated in each experiment in the same way but with the enzyme omitted. This technique was employed because buffered solutions rapidly removed basophilic material from the cytoplasm, but pure water removed basophilia less rapidly; a strict comparison with the control was always necessary to determine how much of the decrease in basophilia could be attributed to the enzyme.

Crystalline ribonuclease (Kunitz, 1940) in 0.04 per cent solution completely removed the inclusion bodies and cytoplasmic basophilia in 15 minutes. This preparation is probably not protease-free but may contain traces of pancreatic proteolytic enzymes.

Protease-free crystalline ribonuclease (Macdonald, 1948) at similar concentration removed cytoplasmic basophilia, but failed in 60 minutes to remove the inclusion bodies. It did, however, alter their appearance, and they stained more eosinophilic and less basophilic than controls.

Crystalline trypsin in 0.04 per cent solution had no effect after 60 minutes' treatment on the staining of trypanosomes, but when combined with an equal amount of the above solution of protease-free ribonuclease the inclusion bodies were removed in 15 minutes. The appearance of the nucleus in all these experiments was unchanged.

Although these results are clear-cut it must be emphasized that the interpretation of individual experiments is a matter largely of judgment owing to the unpredictable behaviour of these enzymes and the fact that controls also showed diminished staining through the action of the distilled water. Yet, in conjunction with the confirmatory tests given below, it can safely be taken that the chief constituents of the inclusion bodies are ribonucleic acid and protein.

Confirmatory tests for the presence of ribonucleic acid and protein in the inclusion bodies

Gram stain.—Henry and Stacey (1943) have shown that the Gram staining material of bacteria is magnesium ribonucleate. The specificity of this test for ribonucleates is doubtful since the reaction mechanism is unknown. The reagents used were crystal violet, and iodine in potassium iodide solution, and the decolorization with absolute alcohol gave the best contrast after 20 seconds. The inclusion bodies were Gram positive.

The Feulgen reaction to distinguish desoxyribonucleic acid from ribonucleic acid was not strictly applicable since the acid digestion necessary for the Feulgen reaction was sufficient to remove all basophilic material from the cytoplasm and the inclusion bodies. The nuclei, however, remained on the slide and gave a faintly positive Feulgen reaction for desoxyribonucleic acid.

Coupling with tetrazotized benzidine (Mitchell, 1942 ; Danielli, 1947).—Of the substances likely to be present in the cell, tyrosine, histidine, and derivatives of pyrimidine are susceptible to attack by a diazonium reagent. The reactivity of tyrosine and histidine can be blocked by benzoylation, and the pyrimidine derivatives, which are not benzoylated, will be stained a reddish brown by the following technique:

Smears are left for 12 hours at room temperature in a solution of 10 per cent (w/v) benzoyl chloride in pyridine, washed well and left for half an hour in 0.05 per cent tetrazotized benzidine in barbiturate buffer at *p*H 9, washed with three changes of buffer and left for 45 minutes in saturated sodium bicarbonate saturated with β-naphthol. The coupling reactions are performed at 0–4° C.

The dye is formed in the nucleus and inclusion bodies of the trypanosomes, indicating the presence of pyrimidine combined with the cell structure ; as vitamins, coenzymes, or other trace substances containing pyrimidine derivatives will have been washed away by this treatment, this is a sensitive cytochemical test for both types of nucleic acid. The pyrimidinium nucleus of antrycide is not attacked by diazonium reagents.

Ultra-violet absorption.—The inclusion bodies absorb heavily in photographs taken at wavelength 2750A. This indicates a concentration of purines and pyrimidines in the inclusion bodies ; this would be a valuable test for nucleic acid but for the pyrimidinium nucleus of antrycide which also absorbs at this wavelength.

Millon's tyrosine reaction.—Unfixed smears were left for 12 hours in Bensley's modification of Millon's reagent (Bensley and Gersh, 1933). The inclusion bodies appeared as dark refractile granules; the outline of the trypanosome could not be seen under the microscope by visual light, but was apparent in photographs taken with a fluorite objective, mercury vapour lamp, and Wood's glass filter with transmission in the 3650A region, which is the absorption maximum of the Millon tyrosine complex (Pollister, 1950).

III. A MODE OF ACTION FOR TRYPANOCIDAL DRUGS WHICH PRODUCE BASOPHILIC INCLUSION BODIES

In the previous paper evidence was put forward that antrycide inhibited the growth of trypanosomes and that it entered the trypanosome and was concentrated in the inclusion bodies. Although previous workers have not drawn the same conclusion for dimidium bromide (Lock, 1950) and suramin (Hawking, 1939; Janscó and Janscó, 1934) there is much evidence that their mode of action is similar. This may be summarized as follows.

1. Chemotherapeutic concentrations are without trypanocidal activity *in vitro*.

2. Normal effective doses may take several days to clear the blood of an infected animal.

3. Chemical estimation of the suspension fluid has either failed to detect absorption of the drug by trypanosomes or detected only minute amounts.

4. Inclusion bodies are produced by all three types of drug after 24 hours at normal effective dose levels.

Strong circumstantial evidence therefore exists that these three drugs are growth inhibitors rather than direct trypanocides and that they enter the trypanosome in small amounts and are concentrated in the inclusion bodies.

It is, of course, by no means certain that the production of inclusion bodies by these drugs is not independent of their trypanocidal activity, and that some other as yet undetected process is not of more fundamental importance, but I believe that the most cogent reason against there being an inhibition of any vital process caused by these drugs is the unimpaired motility of trypanosomes that have absorbed an effective dose. A more satisfactory explanation of their activity is provided by the theory—held generally for some time but best expressed in the work of Caspersson (1950)—that the interaction of protein and ribonucleic acid in the cytoplasm is an essential point of protein synthesis, cell growth, and reproduction; so in the drug-treated trypanosome, growth and reproduction cease when an appreciable amount of the protein or ribonucleic acid is inactivated, as it would be by being removed by the drug from the phase of the cytoplasm to that of the inclusion body.

Two important questions, however, remain.

(i) How can suramin, a molecule with six *negative* charges, act in the same way as dimidium bromide and antrycide which have respectively one and two *positive* charges?

(ii) Why should not all substances that can produce inclusion bodies be trypanocidal?

(i) Some of the less complex nucleoproteins can be considered as salt-like complexes of basic protein and nucleic acid (Greenstein, 1944) and like other salts can *in vitro* be broken down by double decomposition with *either* acids *or* bases. It is most probable that this also can occur in the cytoplasm of trypanosomes. As the molecules of these drugs are large, and the molecules of protein and ribonucleic acid still larger, the resulting complex would be precipitated from the phase of the cytoplasm giving the conditions necessary for a double decomposition of nucleoprotein. Suramin has been shown to form complexes with the basic groups of protein (Wills and Wormall, 1950), dimidium bromide with nucleic acids (Brownlee *et al.*, 1950), and antrycide with acids of high molecular weight (Ormerod, unpublished).

(ii) The charged form of most basic vital stains is in equilibrium at *p*H 7.5 with an uncharged form which enters the trypanosome rapidly. It can also diffuse out again when the outside concentration of the substance is reduced and thereby reverse the reaction between the dye and nucleoprotein. Antrycide, suramin, and dimidium bromide have, however, permanent charges. Antrycide is known to penetrate the cell very slowly, and, as suramin and dimidium bromide produce inclusion bodies at approximately the same rate, they are probably also slow in penetrating the cell. When the external concentration of the drug falls the drug diffuses out of the cell so slowly that the trypanosome may end its normal life span before the processes of growth and division can be resumed. The fundamental difference between the actions of these two classes of substances is that the inclusion bodies produced by vital stains are reversible, and those produced by the trypanocidal drugs treated here are irreversible.

If the cell membrane were thermodynamically " ideal " the rate of diffusion into and out of the cell would be the same, but as a reaction has been demonstrated inside the cell that can fix acidic or basic substances that penetrate the cell membrane, the most probable departure from ideality will favour concentration of these substances in the cell.

Although no inclusion bodies which can be demonstrated in the dry fixed smear have been found in trypanosomes treated with stilbamidine, it is most probable that some similar reaction does in fact occur. Trypanosomes treated with stilbamidine show highly fluorescent granules (Hawking and Smiles, 1941) which resemble the fluorescent granules that appear in the cytoplasm of trypanosomes treated with antrycide. But when stilbamidine treated trypanosomes are stained there are no basophilic inclusion bodies demonstrable. However, in some of the trypanosomes, unstained spaces can be seen, and these give the suggestion that inclusion bodies are present but do not stain with eosin-methylene blue.

Kopac (1945) has postulated a mode of action similar to the one put forward in this paper for stilbamidine against tumour cells ; and inclusion bodies of a similar chemical and histological nature have been demonstrated (Snapper *et al.*, 1947) in myeloma cells from patients treated with stilbamidine, but it is impossible at this moment to fit stilbamidine into the same reaction scheme as antrycide, dimidium bromide, and suramin, for although the hypothesis that I have outlined may describe a mechanism common to the interaction of other drugs and biological systems it is not yet possible to detect it without the appearance of histological changes ; also, when inclusion bodies have appeared it is not possible to prove that they are not subsidiary phenomena unrelated to the main action of the drug. Although it was

demonstrated in the previous paper that antrycide was actually concentrated in the inclusion bodies it may be argued that this is due to the operation of a defence mechanism in the trypanosome removing a noxious substance from the vital parts of the cell; but whether this is so or not, the concentration of protein, ribonucleic acids, and the drug in the inclusion bodies strongly suggests that the drug has upset the normal combination of protein and nucleic acids, which, if one accepts the theories of Caspersson (1950), may be considered sufficient to inhibit the growth of the trypanosome.

Conclusion

The general conclusion derived from this work must be treated as a working hypothesis, since the theoretical background used to interpret experimental results described in this paper cannot be said to have advanced far beyond a state of hypothesis.

Antrycide, dimidium bromide, and suramin inhibit the growth of trypanosomes by splitting the cytoplasmic nucleoprotein into its constituent protein and ribonucleic acids. It is probable that not all the nucleoprotein in the cytoplasm is so affected. Work on other systems (Greenstein, 1944) suggests that only the simpler forms of desoxyribonucleoprotein are held together by purely electrostatic bonds which can be split by acids or bases without denaturation of protein, and the same will probably apply to the ribonucleoprotein involved in the reaction with antrycide. The reaction with neutral red is completely reversible so that in this instance there can be no protein denaturation; if the two reactions are fundamentally the same, as I believe them to be, the conclusion is justified that it is only the simpler nucleoproteins present in the trypanosome which are involved. The more complicated nucleoproteins which might not be broken down by this reaction would account for the basophilia that remains in the cytoplasm after the formation of inclusion bodies either by antrycide or neutral red.

The possibility—advanced by Kopac (1945) to explain the mode of action of stilbamidine on tumour cells—that the trypanocidal drugs treated in this paper act by denaturation of the protein fraction of the cytoplasmic nucleoprotein of trypanosomes, in contrast to the action of non-trypanocidal vital stains which leave the protein undenatured, must be considered. But although this may be a factor in the action of other types of drugs, for reasons which have been stated above, I believe that the action of antrycide, dimidium bromide, and suramin is determined by their possessing electric charges which, once the drugs have penetrated the cell membrane, tend to retain them in the trypanosome, preventing its growth and division.

In this paper the problem of structural specificity of drugs for trypanosomes has not been discussed; this is, however, one of the ways in which it is hoped that further investigation of inclusion body formations may be fruitful.

Summary

1. Basophilic inclusion bodies are produced in trypanosomes in the living animal by effective doses of antrycide, dimidium bromide, and suramin.

2. Similar inclusion bodies are produced *in vitro* by vital stains, and their formation can be observed under the microscope.

3. The relation of these inclusion bodies to the "Volutin" granules, and to basophilic inclusion bodies produced in other biological systems, is discussed.

4. The inclusion bodies produced by antrycide have been shown to contain ribonucleic acid and protein.

5. It is suggested that these drugs inhibit the growth and division of trypanosomes by preventing the normal interaction in the cytoplasm of protein with ribonucleic acid.

6. The activity of antrycide, dimidium bromide, and suramin is connected with the presence in their molecules of permanent charges so that they are retained in the trypanosome; the charged forms of vital stains, on the other hand, are in equilibrium at *p*H 7.5 with uncharged forms which can diffuse rapidly through the trypanosome membrane.

7. The changes produced by the trypanocidal drugs treated here are permanent, whereas those produced by vital stains are reversible.

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