Studies on Serum Protein Changes and Organ Dye Concentrations in Trypan Blue Carcinogenesis.* (28655)

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(Introduced by E. P. Benditt)
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A number of the physiopathologic alterations induced in experimental animals by injection of the diazo dye trypan blue have been attributed, at least in part, to its capacity to bind with protein. Electrophoretic analysis of the serum of dye-treated animals has shown not only changes in the proportions of the various fractions, but also the presence of an abnormal globulin component(1,2,3,4). The teratogenic action of trypan blue in rats and rabbits has been related to alterations in the maternal serum proteins(1). Induction of characteristic neoplasms of the reticuloendothelial system by trypan blue may likewise be associated with changes in serum proteins (2,5). In view of the positive correlations between carcinogenic activity and protein binding capacity which have been found with various other azo dyes(6), it is not surprising that there should be speculation on the role of protein binding in the pathogenesis of the neoplasms induced by trypan blue. In the present study, alterations in serum proteins after one dye injection are compared with those found later in animals developing dye induced neoplasms and concentration of protein bound dye in the serum and reticuloendothelial organs is evaluated and related to degree of immediate cellular response and the eventual development of neoplasms.

Materials and methods. Wistar strain rats weighing 200-300 g were housed in individual cages and fed a diet of Purina Laboratory Chow with a supplement†. Water was available *ad lib,* and fresh greens were fed twice weekly.

Two groups of healthy untreated animals of the same age and weight as the experimental groups were bled for determination of normal protein values (Table I). Following a single subcutaneous injection of an aqueous solution of trypan blue (National Aniline Co., Lot #16944), serum for protein determinations was obtained from groups of animals at the intervals indicated in Table I. Each animal was bled from the tail vein only once or twice during the experimental period with removal of not more than 1 ml of blood. Animals developing histiocytic tumors of the liver from 1-3 months after a series of biweekly dye injections were anesthetized and killed by bleeding from the aorta.

Total proteins were determined using Biuret reagent according to the method of Wolfson and Cohn(7). Serum protein fractions were measured by paper electrophoresis in a Spinco inverted V-type cell. The strips were exposed for 17 hours at 4 ma. using a barbital buffer at pH 8.6 and ionic strength 0.075. The strips were scanned, after staining with Spinco dye B-1, in a model RB Analytrol, using a B-5 cam. Boric-Borax Buffer, pH 9.2, was also used in an effort to obtain better separation of the serum globulin fractions, but the results were inferior to those obtained with the barbital buffer(8).

The amount of trypan blue bound to cellular protein in the liver, spleen, lymph nodes and serum was measured using a modification of the method of Judah and Willoughby(9). Following homogenization of the tissues in a Waring blendor with buffered saline, pH 7.0 as the medium, the protein was precipitated from 1 ml aliquots of serum and liver homogenates and 5 ml aliquots of spleen and lymph node homogenates with an equal volume of 10% trichloracetic acid. To remove free dye and lipids, the precipitate was then extracted once with 50% aqueous ethanol at room temperature followed by absolute alcohol extrac-

* This investigation was supported in part by a grant from Nat. Cancer Inst., Nat. Inst. Health.

† Ground whole wheat 67.5%; casein, Tech. 15%; skim milk powder 7.5%; NaCl .75% (iodized); calcium carbonate 1.5%; melted fat 6.75%; fish oil (vit. A-D concentrate) 1.0% KI solution.

tions for 5 minutes at 70°C until the supernatant was clear and colorless. The dye was then removed from the protein precipitate by incubating at 70°C in 25% aqueous pyridine for 40 minutes or until elution was complete. The color of the supernatant was read in the Coleman Junior spectrophotometer at 600 mu against a blank prepared from normal liver, and the dye concentration calculated from a previously prepared curve.

TABLE I. Total Proteins and Fractions in Serum of Normal and Trypan Blue Treated Animals.

Experimental animals	No. of animals	Total protein (g %)	Albumin	α^1 globulin	Unknown component	α^2 globulin	β globulin	γ globulin
			(%)					
Normal controls	23	6.6 ± .39†	41.5 ± 3.57	17.4 ± 1.58	6.9 ± 1.34	7.2 ± 1.11	16.9 ± 4.07	9.7 ± 1.52
Trypan blue treated								
1 day*	7	5.9 ± .22	35.0 ± 5.36	19.4 ± 1.47	9.5 ± 1.40	8.2 ± 1.64	19.0 ± 3.51	8.5 ± 1.44
2 days	10	6.2 ± 1.29	31.2 ± 5.11	18.5 ± 1.86	10.2 ± 1.92	10.0 ± 5.22	19.5 ± 3.21	10.2 ± 2.86
3 "	10	6.1 ± .72	29.9 ± 4.29	19.8 ± 2.23	10.0 ± 1.40	8.5 ± 1.07	20.7 ± 3.03	10.8 ± 1.83
4 "	11	5.3 ± 1.10	30.2 ± 5.11	19.5 ± 3.11	8.2 ± 1.40	8.1 ± 1.62	18.9 ± 2.54	14.7 ± 4.10
7 "	8	6.1 ± .56	31.1 ± 6.67	16.4 ± 2.19	6.9 ± .73	6.5 ± 1.35	17.3 ± 2.19	21.5 ± 9.31
9 "	7	5.0 ± .52	29.0 ± 9.69	18.7 ± 3.13	7.5 ± 1.13	6.3 ± 1.05	20.9 ± 2.36	17.3 ± 6.03
Normal controls	24		48.1 ± 3.36	16.7 ± 3.14	6.3 ± 1.39	4.3 ± 1.30	14.9 ± 1.91	9.3 ± 2.27
Trypan blue treated Histiocytic tumors‡	20	3.7 ± .97	41.4 ± 9.49	14.1 ± 5.07	6.6 ± 1.94	6.7 ± 1.81	18.0 ± 3.54	12.8 ± 4.70

* In all cases, days after a single injection of 20 mg/100 g trypan blue.
† Mean and standard deviation.
‡ Tumors appeared from 28-100 days after a series of 15 biweekly injections of 10 mg/100 g trypan blue.

Results. Following a single injection of dye the amount of total serum protein was reduced throughout the 9 day observation period (Table I). The most significant decrease, found on the ninth day ($p > .001$), was largely due to a reduction in the albumin fraction ($p > .001$). During this same period, all the globulin fractions increased with the most marked change in the gamma globulin.

Histiocytic tumors of the liver developed in response to prolonged injection of dye. The total serum proteins of these tumor bearing animals, measured one to 3 months after the last injection, were reduced but there was no notable change in the proportion of the protein fractions (Table I).

Yamada has described an abnormal band, that was consistently visible between α^1 and α^2 globulin in the electrophoretic pattern of serum obtained from rats 1-2 days after treatment with trypan blue(4). In the present study a similar band was observed, but was seen not only in the serum of dye treated animals, but also of many controls. However, dye treatment increased the incidence of this fraction from 15% in untreated animals to 50% in the experimental group. To assess this fraction quantitatively, its concentration on the electrophoretic strips was measured in the analytrol (Table I). In those cases in which the band was not visible, the area corresponding to its characteristic location was measured arbitrarily. The mean concentration of this component derived from values on all animals was found to be increased on the first day after dye injection and remained elevated for as long as 4 days (Table I). Analysis of the serum of tumor bearing animals revealed this band in only about 20% of the cases, with no significant increase in either incidence or concentration over that of untreated controls. No other abnormal protein components were found in the serum of these animals.

The dye concentration in the serum, liver, and spleen following injection of trypan blue is shown in Figure 1. The dye appeared to be completely bound to protein as evidenced

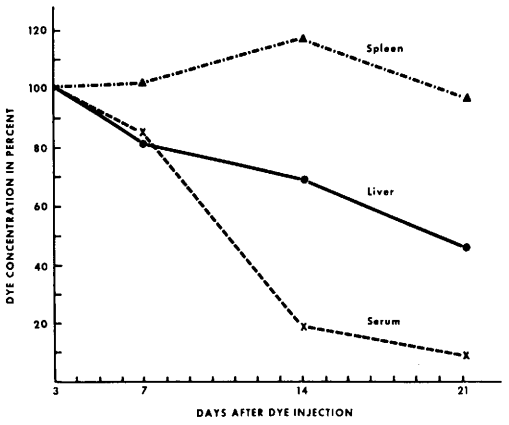

FIG. 1. Dye concentration in the serum liver and spleen following one injection of 5 mg/100 g of trypan blue. Each point, representing the mean of 3 animals is expressed as a percentage of the 3-day value. Mean dye concentrations in γ/ml: Spleen—3 days—80 $\pm$ 15.3; 7 days—82 $\pm$ 3.9; 14 days—94 $\pm$ 24.3; 21 days—77 $\pm$ 9.4. Liver—3 days—657 $\pm$ 112.2; 7 days—545 $\pm$ 109.9; 14 days—453 $\pm$ 149.4; 21 days—307 $\pm$ 77.4. Serum —3 days—93 $\pm$ 26.3; 7 days—80 $\pm$ 17.0; 14 days —17 $\pm$ 4.7; 21 days—8 $\pm$ 1.5.

by a blue precipitate and a colorless supernatant after precipitation with trichloracetic acid and extraction with ethanol. Although the organs were not perfused, the animals were killed by bleeding from the aorta and it is evident that serum dye concentration steadily decreased presumably as the injection site was cleared of dye, while the organs retained high levels for a longer period. The concentration of dye in the spleen as well as the axillary lymph nodes remained relatively constant throughout the 21 day observation period, in contrast to a 50% reduction in the liver during the same time.

Discussion. Our observations of a reduction in total protein and albumin and the appearance of an abnormal band between α^1 and α^2 globulin are in agreement with the findings of others(3,4). Yamada has suggested that the abnormal band which he observed only in the serum of trypan blue treated rats resulted either from a change in the electron charge of proteins, synthesis of an abnormal protein, or its release from storage cells. It is of interest that Paoletti observed this new protein fraction not only in rats treated with trypan blue but also with the hepatic carcinogen, ethionine(3). Our observation of this band in the serum of a number of control animals with an increase in both incidence and concentration suggests that the dye instead of inducing the synthesis of abnormal proteins stimulated the production or release of a protein fraction normally present in undetectable amounts. Alternately, the dye-induced alterations in the proportions of the various fractions may have simply rendered the band more visible. Since we found that the increase in this fraction was directly related to the dye administration it is not surprising that its frequency was not increased in animals succumbing with tumors, since several months had elapsed since their last injection. Although total serum proteins were reduced both in the tumor bearing animals and in those given only one dye injection, different mechanisms appeared to be involved. The hypoproteinemia of the former group was attributed to the advanced stage of neoplasia with extensive liver involvement(5). In the acutely dye-treated group, on the other hand, the disproportionate reduction in the albumin fraction was significant and was thought to be related to the preferential binding of the dye with albumin, which has been demonstrated both *in vivo* and *in vitro*(2,10). The relationship, if any, between these acute changes in the serum proteins and the reticuloendothelial neoplasms that eventually developed is not clear. In this connection Gillman was unable to find any direct correlation between serum protein composition and reticulosis in the livers of trypan blue treated rats(2). However, alterations in the serum proteins have been produced by the feeding of several carcinogenic azo dyes and the development of embryonic malformation has been linked to disturbances of the maternal serum proteins in trypan blue treated rats (1,11).

It appears that further studies of the interaction of trypan blue with cellular protein might contribute to an understanding of its carcinogenic activity. In this connection, the data on organ dye concentration are of interest. Although the dye is found to be bound to protein in the liver, spleen and lymph nodes, as well as in the blood, reticulo-

endothelial neoplasms develop only in the liver. That carcinogenesis is not merely a function of dye concentration is indicated by the fact that although the amount of dye per gram of tissue is highest in the spleen, no primary tumors occur in that site. Neither is reticuloendothelial cell hyperplasia *per se* directly related to the development of neoplasms, for cellular proliferation is marked in both the spleen and lymph nodes. The rapid fall in dye concentration in the liver suggests that it has a different method of handling the dye. It seems possible that the specific carcinogenic action of trypan blue on the reticuloendothelial cells of the liver may be related to a unique capacity which this organ possesses for disposing of the dye and free dye or a metabolite or degradation product may, through an interaction with protein, play a role in the initiation of carcinogenesis. Thus, the mechanism of action of trypan blue may be analogous to that of other structurally similar carcinogenic azo dyes such as p-dimethylaminoazobenzene, in which metabolic breakdown of the dye and protein binding are regarded as important steps in the causation of hepatic tumors(12).

Summary. A single injection of trypan blue produced a prompt reduction in total serum proteins due largely to a decrease in the albumin fraction. The hypoproteinemia, with a reduction in all fractions, found in animals bearing dye induced reticuloendothelial tumors was attributed to the advanced stage of neoplasia. An "abnormal" band between α^1 and α^2 previously described in the serum of rats only after trypan blue treatment was seen in the present study in the serum of untreated animals, but was quantitatively increased after the dye injection. The incidence and concentration of this band were not increased in tumor bearing animals. Evaluation of the degree of cellular response and the concentration of protein bound dye in the liver, spleen and lymph nodes following one dye injection indicated that neither could be correlated with the eventual development of reticuloendothelial neoplasms in the liver. However, the relatively rapid fall in the concentration of protein bound trypan blue in the liver led to speculation that tumorigenesis in this organ might be due to release of free dye or formation of metabolic breakdown products.

The authors are indebted to Miss Donna Gorder for skilled technical assistance.

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Received May 17, 1963. P.S.E.B.M., 1963, v114.