EFFECTS OF ARSENIC-AZOPROTEINS ON MOUSE LYMPHOMA CELLS IN VIVO*

With Observations on the Effects of Other "Anti-Lymphoma" Agents, and on the Susceptibility to These Effects of Lymphoma Cells of Various Types

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Plates 44 and 45

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Previous studies have shown that immune serums made with mouse lymphoma cells as antigens can act powerfully and to some extent specifically *in vivo* on the cells of several mouse lymphomas, though none has proved powerful enough to overcome advanced lymphomatosis in susceptible mice (1). In the initial tests of the work now to be reported, conjugates were made by coupling diazotized arsanilic acid (4-arsonophenyldiazotate) with an anti-Lymphoma 6C3HED–immune rabbit serum, on the assumption that the attached arsenic might enhance the effects of the immune globulins on lymphoma cells of this type *in vivo*. In several experiments, arsenic-azo–immune globulin preparations indeed acted much more powerfully upon Lymphoma 6C3HED cells *in vivo* than did the immune globulins alone, bringing about the complete regression of established 6C3HED lymphomas in susceptible ZBC mice without harming the latter perceptibly, while untreated control animals regularly died with lymphomatosis. But further experiments promptly disclosed that arsenic-azo preparations made with a variety of other proteins (*e.g.*, bovine albumin, horse or bovine globulin, ovalbumin, and casein) were also effective against the proliferating lymphoma cells *in vivo*—and, so far as this could be judged, fully as effective as the immune conjugates—while a variety of inorganic and organic arsenicals (including arsanilic acid and 4-arsonophenyldiazotate) were essentially devoid of such effect. So, too, the proteins, and simple mixtures of arsanilic acid and the proteins, had no such effect on Lymphoma 6C3HED cells *in vivo*, and this proved true as well of various sulfurazoproteins and of a number of "chemotherapeutic" agents—*viz.*, ameth-

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opterin, chlorambucil, 6-mercaptopurine, azaserine, 8-azaguanine, 6-azauracil, thioTEPA, 5-fluorouracil, and DON, each given in maximal tolerated amounts. The findings will now be given in detail, and something will be said further on of their implications.

Materials and Methods

The methods were similar to those previously employed (1, 2). In brief, Lymphoma 6C3HED cells, and those of other mouse lymphomas also, were implanted in the subcutaneous tissues of both groins of inbred susceptible mice, with injection of the test materials intraperitoneally into arbitrarily selected groups of animals—usually after the lymphoma cells had proliferated to form palpable masses (*i.e.*, 7 days or more following implantation)—with careful observation thereafter of the course of the lymphomas and the state of their animal hosts.

For tests with Lymphoma 6C3HED cells for example, 0.5 million or more of the 6C3HED cells were implanted in each groin of susceptible hosts—either ZBC mice supplied by Dr. John Bittner or C3H/Jax mice procured from The Jackson Memorial Laboratory, Bar Harbor. Following such implantations, palpable lymphomas regularly appeared in the groins of untreated hosts, usually after 5 to 7 days. In untreated ZBC hosts, the lymphomas enlarged progressively and regularly brought about death, usually within 30 days. Some hours or days following the implantations (usually after 7 days or more, when subcutaneous lymphomas approximately 1.0 cm. or more across had become palpable in both groins), the materials to be tested were injected intraperitoneally into groups of 4 or more mice. The test animals, together with untreated controls, were then carefully followed, often with daily weighings and observations to learn whether the injected materials were toxic, and with frequent chartings of the palpated tumor masses, and gross and microscopic postmortem studies when indicated to determine the effects of the injected materials on the lymphoma cells *in vivo*. Similar tests were made with Lymphoma E9514 cells in C3H/Jax mice, with Lymphoma AKRL1 and L4946 cells in AKR/Jax mice, and with L1210 cells in D2BC mice.

Preparation of Arsenic-Azoproteins.[1]—4-arsonophenyldiazotate was made by a modification of the procedure of Eagle and Vickers (3), as follows: 5 gm. arsanilic acid (Eastman) was dissolved in 50 ml. normal HCl, and to the solution 75 ml. water was added. The solution was then cooled to 0°C. in an ice bath, and 23 ml. molar $NaNO_2$ (0°C.) was rapidly added, the solution becoming pale yellow. Sufficient 2 N NaOH was next added to bring the pH, as determined with the glass electrode, to the desired value, usually between 7.4 and 9.5, the color of the solution darkening as result. 8 ml. of 0.5 molar phosphate buffer (at the desired pH) was next added, and the volume adjusted to 200 ml. with H_2O.

Conjugation with one or another of the several proteins was carried out as a rule at 0°C. and pH 9.5 for periods of 0.5 to 8 minutes; occasionally at 22°C., with pH between 7.4 and 8.5 for periods of 5 to 45 minutes. In the routine preparation of 4-arsonophenylazo—horse globulins, for example, the diazotate was mixed in equal parts with normal horse serum (generously supplied by the Bureau of Laboratories, New York City Department of Health), with pH adjusted as required. After conjugation had proceeded during the selected interval, an equal volume of saturated ammonium sulfate was added to the mixture, with result in immediate precipitation of the dyed protein. The pH of the mixture was adjusted to 5.4 with normal HCl, and the mixture was kept 15 to 30 minutes at 37°C. The yellowish precipitate, recovered by means of centrifugation, was suspended in water, and formed a reddish brown solution when the pH was brought to 7.4 or above; samples of this solution became yellowish and somewhat opalescent upon reacidification. The reddish brown solution (pH 7.4), was

[1] I am indebted to Dr. Walther Goebel and Dr. Donald Melville for advice in this matter.

dialyzed in successive changes of 0.9 per cent NaCl solution during 3 days at 4°C., the dialysate being then free from sulfate ions as determined with $BaCL_2$; next it was filtered through a Seitz EK disc, the volume of the reddish brown filtrate being approximately equal to that of the serum initially employed. Samples, cultured on agar and in enriched broth, regularly proved sterile.

Arsenic–azoglobulins were also made by first precipitating the globulins from horse serum or rabbit serum with ammonium sulfate at half saturation, dialyzing against 0.9 per cent NaCl solution, and then coupling the sulfate-free globulins with 4-arsonophenyldiazotate, with recovery of the globulins as described above. 4-arsonophenylazo-bovine albumin (Armour), 4-arsonophenylazo-bovine gamma globulin (Armour), and 4-arsonophenylazo-casein (Nutritional Biochemicals Co.) were made as described above with solutions of the respective proteins, usually in 4 or 8 per cent concentrations, with acidification to pH approximately 5.0 immediately after conjugation, the uncombined diazotate being removed by dialysis in running tap water. Similar preparations were made with solutions of gelatin (Difco), gliadin (Mann Research Laboratories), twice crystallized ovalbumin (Nutritional Biochemicals Co.), and bovine glycoprotein VI (Nutritional Biochemicals Co.). Arsenic-azopeptone (Difco) and arsenic-azohistidine (Nutritional Biochemicals Co.) were also prepared similarly except that alcohol was used to precipitate the conjugates. For control purposes, tests were made with sulfur-azoproteins prepared by conjugating the proteins with diazotized sulfanilic acid under conditions similar to those employed in the preparation of the arsenic-azoproteins.

Assays of the Conjugates.—Using an adaptation of the method of Krieckhaus as modified by Low (4), Mr. Walter Matusiak, working under the supervision of Dr. C. J. Umberger, Department of Forensic Medicine, Post Graduate School of Medicine, New York University, determined the arsenic content of a number of the conjugates. Determinations of protein in these specimens were made by Miss Adela Leibman, Research Associate, Hospital for Special Surgery, New York, using the micro Kjeldahl procedure of Meeker and Wagner (5).

Another means of assay was more extensively employed. Observation with the naked eye showed that conjugates made by coupling proteins with diazotized arsanilic acid for ½ minute at pH 9.5, or for 5 minutes at pH 7.4, gave solutions with relatively faint color, while progressively more deeply colored solutions were obtained if the couplings were allowed to proceed longer. Spectrophotometry, initially employed by Mr. Arthur Jaffe, made plain that the optical densities (hereinafter OD) of solutions of arsenic-azoproteins prepared in standardized ways were remarkably uniform. For example, a number of preparations made by mixing various lots of horse serum or rabbit serum with 4-arsonophenyldiazotate at pH 9.5 for 30 seconds, with recovery of the dyed azoglobulins as described above, had optical densities of approximately 0.34 when tested in the Coleman instrument with light of 400 mμ, in dilution 1:50, pH 7.4. Prolonging the period of coupling to one minute at pH 9.5 gave preparations with optical densities of approximately 0.39, and coupling for 2 minutes and 8 minutes at pH 9.5 provided preparations with optical densities of 0.57 and 0.66, respectively. In general, the former preparations proved notably effective against established 6C3HED cells growing in susceptible hosts when they were given in amounts of 0.5 to 2.0 cc. per mouse per day on 3 to 5 successive days, as will be brought out further on, and they were devoid of perceptible toxic effects on the mouse hosts.

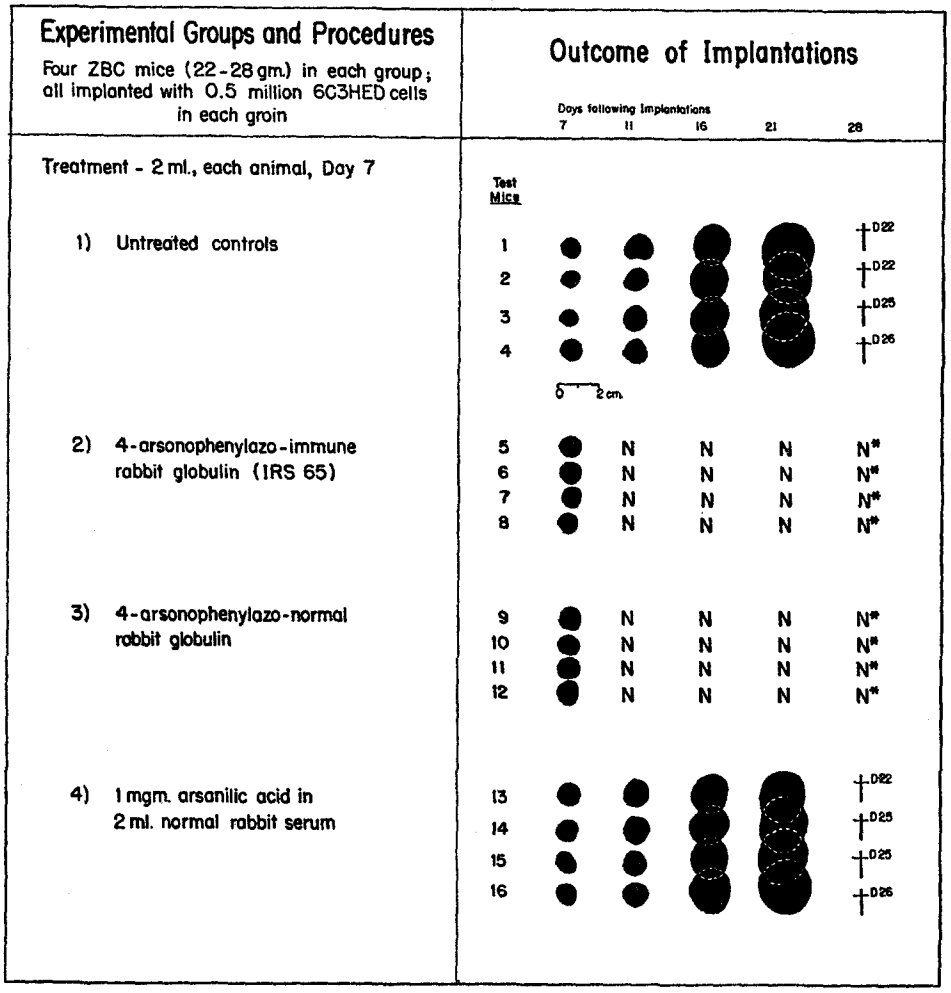
Inhibitory Effects of Arsenic-Azoproteins on Lymphoma 6C3HED Cells in Vivo; Tests with Other Substances

Charts 1 and 2 show the outcome of three experiments in which tests were made with arsenic–azo conjugates prepared respectively with immune rabbit

CHART 1. Effects on Lymphoma 6C3HED cells *in vivo* of arsenic-azoproteins made with (*a*) anti-lymphoma immune and (*b*) normal rabbit serums, respectively.

The arsenic-azoproteins were made by mixing 50 ml. of 4-arsonophenyldiazotate with 50 ml. of each serum at 0°C., pH 9.5 for 1 minute, the proteins being then precipitated with ammonium sulfate at half-saturation, pH 5.4, suspended in 50 ml. H_2O and dialyzed against 0.9 per cent NaCl solution. The immune globulin and normal globulin conjugates, dissolved in 50 ml. 0.9 per cent NaCl solutions, pH 7.4, had optical densities (hereinafter OD) as measured with the Coleman spectrophotometer at 400 mμ, dilution 1:50, of 0.39 and 0.41, respectively. They were injected intraperitoneally as indicated; so too in all subsequent experiments.

To conserve space, only the tumor in the right groin of each test mouse is here charted; the opposite side was comparable in every instance. So too in Chart 2 which follows.

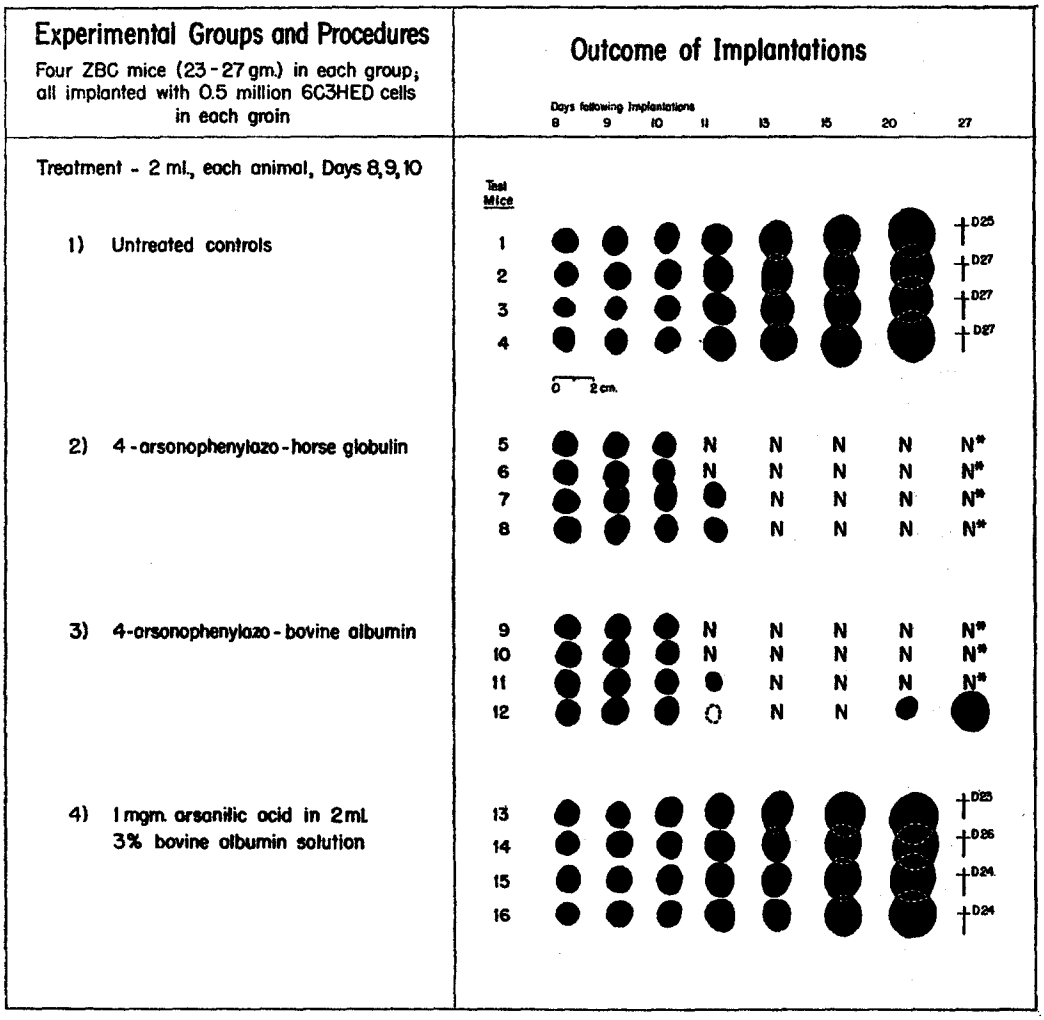


* N = negative. All mice of groups 3 and 4 maintained their respective weights (±2 gm.) during the charted period; they remained fleshy and lively throughout an observation period of 120 days, and all proved fully susceptible to reimplantation with 6C3HED cells made on the 121st day. Day 26, etc. = died on the 26th day, etc.

In other experiments Lymphoma 6C3HED cells implanted as above in more than 600 untreated ZBC mice have regularly grown progressively and brought about death of the animals, with signs of generalized lymphomatosis, usually within 30 days. Arsanilic acid in maximal tolerated amounts (1 to 2 mg./mouse/each of 3 to 6 successive days) in mixture with normal horse serum or globulin, normal or immune rabbit serum or globulin, or in 0.9 per cent NaCl solution, has proved entirely devoid of effect on 6C3HED lymphoma cells in 190 of 194 ZBC mice.

CHART 2. Effects on Lymphoma 6C3HED cells *in vivo* of arsenic-azoproteins made with (*a*) horse globulin and (*b*) bovine albumin.

Globulin was precipitated from 50 ml. normal horse serum with half-saturated ammonium sulfate, centrifuged, suspended in water, and dialyzed against 0.9 per cent NaCl solution until sulfate-free; then mixed in equal parts with 4-arsonophenyldiazotate pH 7.4, 0°C. 30 minutes, with recovery of the dyed protein as previously described. OD (400 mμ, pH 7.4, dilution 1:50) = 0.29. The arsenic-azo-bovine albumin was prepared by mixing 100 ml. of 3 per cent bovine albumin (Armour-fraction V) with 100 ml. 4-arsonophenyldiazotate at pH 9.5 for 2 minutes; the dyed protein was then precipitated with ammonium sulfate (full saturation, pH 4.8) and recovered in a volume of 50 ml. OD (400 mμ, pH 7.4, dilution 1:50) = 0.35.



*N = negative throughout observation period (63 days). Mouse 12 died with a large subcutaneous lymphoma in right groin and signs of generalized lymphomatosis on day 50. The other animals in groups 2 and 3 were lively and gained weight (1 to 3 gm.) throughout the observation period.

globulin (1), normal rabbit globulin, normal horse globulin, and bovine albumin, as also with arsanilic acid in simple mixture with normal rabbit serum and bovine albumin respectively. It can be seen that all the arsenic-azoproteins brought about complete and permanent regression of the 6C3HED lymphomas in every ZBC animal save one, while the simple mixtures of arsanilic acid and proteins were devoid of effect. In several similar experiments, conjugates were made under identical conditions with one or another of several batches of pooled anti-6C3HED immune rabbit serum and with various batches of pooled normal rabbit serum. As judged by the regularity and promptness with which established 6C3HED lymphomas regressed in mice given the respective conjugates in various amounts, the preparations made with normal rabbit globulins were as potent as those made with immune rabbit globulins—see Chart 1 for an example. In 41 additional experiments similar to those of Charts 1 and 2, 26 conjugates made by mixing 4-arsonophenyldiazotate in equal parts with horse or rabbit serum, or with solutions of rabbit or horse or bovine globulins, or bovine albumin, regularly brought about permanent regression of established 6C3HED lymphomas in ZBC mice, whereas the lymphomas regularly grew progressively to bring about death of the hosts, usually with signs of generalized lymphomatosis, in more than 600 untreated control animals. Furthermore, in two experiments a preparation of arsenic-azo–mouse globulin made by coupling mouse serum with diazotized arsanilic acid at pH 9.5 for 2 minutes and separating the dyed globulins as previously described, also brought about the complete and permanent regression of established (7 day) 6C3HED lymphomas in ZBC mice.

The results of extensive tests in two experiments with simple mixtures of arsanilic acid and proteins, and with various organic and inorganic arsenicals including 4-arsonophenyldiazotate, are given in Table I, together with the results of tests with nine arsenic-azo- and 4 sulfur-azoprotein preparations made under a variety of conditions.

It can be seen (Table I) that arsanilic acid, given in maximal tolerated amounts in mixture with horse globulin, bovine albumin, bovine gamma globulin, sodium caseinate, or ovalbumin, had no influence on the lymphoma cells *in vivo*; the same was true of arsanilic acid in saline solution, and of 4-arsonophenyldiazotate, potassium arsenite, potassium arsenate, arsenic acid, carbarsone, and mapharsen, each given in maximal tolerated amounts; so too of the proteins given as such. The findings recorded in Table I are largely but not wholly representative of the outcome of numerous tests made throughout the work with arsanilic acid and the other arsenicals employed. For regression of established 6C3HED lymphomas was observed in 4 of 194 mice given repeated injections of 2.0 ml. of arsanilic acid in protein or NaCl solutions (0.5 or 1.0 mg..ml.), the animals in three of the exceptional instances receiving the larger amount and showing hyperactivity and weight loss and other signs of arsenic toxicity during or immediately following the course of injections. The same was true of one of 44 mice given mapharsen, the quantity given in this exceptional instance being almost lethal for the animal in question (inducing marked signs of arsenic toxicity from which, however, the animal managed to recover) and killing the other 3 mice of its group. No instance of regression was observed in numerous mice given near lethal amounts of the other arsenicals

including 4-arsonophenyldiazotate. The findings with arsanilic acid and mapharsen, although exceptional, make plain, however, that toxic amounts of these arsenicals can sometimes bring about regression of established 6C3HED lymphomas in susceptible mice.

Five of the 9 arsenic-azoprotein preparations tested in the experiments of Table I—those made by conjugating 4-arsonophenyldiazotate with horse globulin, bovine albumin, bovine gamma globulin, sodium caseinate, and ovalbumin, respectively, were notably effective in overcoming established Lymphoma 6C3HED cells in susceptible mice, as the tabulated data show; while the four other arsenic-azoprotein preparations—those made with bovine glycoprotein, gliadin, lactalbumin, and gelatin, respectively—had little or no such effect. The former, as can be seen from the table, besides being deeply colored (OD: 0.36 to 0.70), contained relatively large quantities of protein (21.0 to 40.2 mg./ml.) and also relatively large quantities of arsenic (0.42 to 1.11 mg./ml.); the latter, by contrast, although being also deeply colored (OD: 0.34 to 0.70) and containing large concentrations of arsenic (0.74 to 0.89 mg./ml.), had much lower concentrations of protein (1.1 to 8.5 mg./ml.). In this relation it should be noted that a darkly colored conjugate made with peptone and containing 2.54 mg. arsenic/ml. was highly toxic for ZBC mice but devoid of effect on Lymphoma 6C3HED cells *in vivo* when given in maximal tolerated amounts (see footnote Table I); also that a solution of yellowish crystals (40 mg./ml.) procured from a mixture of 4-arsonophenyldiazotate and histidine, was devoid of effect on the lymphoma cells *in vivo*; and that the four sulfur-azoproteins, although deeply colored and containing protein in concentrations of 22.8 to 27.1 mg./ml., were likewise devoid of effect on the lymphoma cells *in vivo*. These findings also deserve consideration in relation to a larger experience with such materials. For although none of the numerous sulfur-azoproteins repeatedly tested throughout the work ever displayed any ability to overcome Lymphoma 6C3HED cells *in vivo*, in several additional experiments, colored conjugates made with bovine glycoprotein, lactalbumin, or gelatin under different conditions (*e.g.*, at pH 7.4 for periods of 30 minutes or longer) acted powerfully against the lymphoma cells *in vivo*, whereas the corresponding preparations of Table I did not, as already stated; unfortunately these more active preparations were used up before protein and arsenic determinations could be made on them.

It is noteworthy that the test mice of Table 1 tolerated much more arsenic when this was given as arsenic-azoprotein or carbarsone than was the case when the arsenic was administered as potassium arsenite, potassium arsenate, arsenic acid, or mapharsen.

The data of Table 1 suggest that only those conjugates containing relatively large quantities of precipitable protein as well as relatively large quantities of bound arsenic were notably active against Lymphoma 6C3HED cells *in vivo*. More will be said about this in the discussion. Meanwhile several observations will be cited which indicate that the proteins here employed were altered upon conjugation with 4-arsonophenyldiazotate.

In this latter relation, it may be recalled that Landsteiner (6) has shown that the antigenic specificity of proteins is regularly altered when these are coupled with certain diazotates, while from the work of Eagle, Smith, and Vickers (7) it can be inferred that the conditions of conjugation and also the character of the proteins determine the degree of alteration undergone by proteins conjugated with such substances. Several observations made during the course of the present work suggest that the protein components of the arsenic-azoproteins had been notably altered. For example, some of the arsenic-azogelatin preparations "jelled" when they were chilled, while others, made under different conditions, failed to do so, this being the case with the gelatin conjugate of Table I. In other experiments, conjugates made with normal guinea pig serum and 4-arsonophenyldiazotate in proportions of 2:1 or 3:1, at pH

TABLE I

Tests for Effects of (a) Mixtures of Arsanilic Acid and Proteins, (b) Various Inorganic and Organic Arsenicals, and (c) Several Arsenic-Azo- and Sulfur-Azoproteins on Established 6C3HED Lymphomas in ZBC Mice

Experimental groups and test materials 4 ZBC mice (22–28 gm.) in each group—all implanted in each groin with 0.5 million lymphoma 6C3HED cells on Day 1			Outcome					
Intraperitoneal injections, each mouse: days 8, 9, 10, 11, 12, 13	Protein, *mg./ml. (calculated)*	As, *mg./ml. (calculated)*	Growths in four test mice* Days 8	12	15	18	22	Subsequent course
1. Nil—untreated controls	—	—	++++	++++	++++	++++	++++	GL—died—days 23, 23, 26, 27
2. Arsanilic acid, 0.5 mg./ml., in horse globulin, 2.0 ml.	40	0.17	++++	++++	++++	++++	++++	GL—died—days 24, 25, 25, 27
3. Arsanilic acid, 0.5 mg./ml., in 4 per cent bovine albumin, 2.0 ml.	40	0.17	−++++−	++++	++++−	++++	++++	" " " 25, 25, 28, 28
4. Arsanilic acid, 0.5 mg./ml., in 4 per cent bovine gamma globulin, 2.0 ml.	40	0.17	++++	++++	++++	++++	++++	" " " 28, 28, 28, 29
5. Arsanilic acid, 0.5 mg./ml., in 4 per cent sodium caseinate, 2.0 ml.	40	0.17	++++	++++	++++	++++	++++	" " " 23, 23, 26, 27
6. Arsanilic acid, 0.5 mg./ml., in 4 per cent ovalbumin, 2.0 ml.	40	0.17	++++	++++	++++	++++	++++	" " " 23, 24, 24, 26
7. Arsanilic acid, 0.5 mg./ml., in 0.9 per cent NaCl, 2.0 ml.	—	0.17	++++	++++	++++	++++	++++	" " " 25, 26, 26, 26
8. 4-arsonophenyldiazotate 1:80, 2.0 ml.	—	0.099	++++	++++	++++	++++	++++	GL—died—days 25, 25, 26, 27
9. " " 1:160, 2.0 ml.	—	0.049	++++	++++	++++	++++	++++	" " " 21, 23, 23, 27
10. Potassium arsenite, 0.08 mg./ml., in 0.9 per cent NaCl solution, 2.0 ml.	—	0.047	++++	++++	++++	++++	++++	GL—died—days 23, 23, 23, 28
11. Potassium arsenate, 0.5 mg./ml., in 0.9 per cent NaCl solution, 2.0 ml.	—	0.21	++++	++++	++++	++++	++++	" " " 27, 29, 30, 32
12. Arsenic acid, 0.25 mg./ml., in 0.9 per cent NaCl solution, 2.0 ml.	—	0.13	++++	++++	++++	+++	++++	" " " 23, 23, 27, 27
13. Carbarsone, 4 mg./ml., in 0.9 per cent NaCl solution, 2.0 ml.	—	1.15	++++	++++	++++	++++	++++	" " " 25, 26, 27, 28
14. Mapharsen, 0.3 mg./ml., in 0.9 per cent NaCl solution, 2.0 ml.	—	0.096	++++	++++	++++	++++	++++	" " " 23, 23, 24, 26

	Conditions of conjugation		Protein, *mg./ml.* (*determined*)	As, *mg./ml.* (*determined*)	OD—400 mμ, pH 7.4, 1:50						
	pH	*min.*									
15. Nil—untreated controls			—	—		++++	++++	++++	++++	++++	GL—died—days 25, 27, 28, 31
16. 4-arsonophenylazo-horse globulin, 2.0 ml.	9.5	1.5	40.2	0.71	0.46	++++	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0	Survived—60 days
17. " -bovine albumin, 1.0 ml.	9.5	1.5	29.2	0.53	0.36	++++	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0	" " "
18. " - " gamma globulin, 1.0 ml.	9.5	2	27.0	0.42	0.52	++++	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0	" " "
19. " -sodium caseinate, 1.0 ml.	8.0	30	21.0	1.11	0.70	++++	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0	" " "
20. " -ovalbumin, 0.5 ml.	8.0	40	26.9	0.61	0.66	++++	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0	" " "
21. " -bovine glycoprotein, 0.5 ml.	8.5	20	8.5	0.86	0.70	++++	0 0 0 0	0 0 0 0	+0 0 0	+++ 0	1 survived—60 days; 3 died—GL—days 28, 31, 35
22. " -gliadin, 1.0 ml.	8.5	15	3.3	0.74	0.68	++++	++++	++++	++++	++++	Discarded—day 23
23. " -lactalbumin, 2.0 ml.	9.0	30	2.6	0.80	0.35	++++	++++	++++	++++	++++	" " "
24. -gelatin, 1.0 ml.	9.5	2	1.1	0.89	0.34	++++	++? ?	++++	++++	++++	GL—died—days 25, 28, 31, 31
25. 4-sulphonophenylazo-horse globulin, 1.0 ml.	9.5	1.5	27.1	—	0.81	++++	++++	++++	++++	++++	GL—died—days 26, 28, 28, 29
26. " -bovine albumin, 1.0 ml.	9.5	2	25.5	—	0.81	++++	++++	++++	++++	++++	" " " 22, 25, 25, 25
27. " - " gamma globulin, 1.0 ml.	9.5	2	22.8	—	0.89	++++	++++	++++	++++	++++	" " " 28, 28, 29, 29
28. " -caseinate, 2.0 ml.	9.5	30	23.7	—	0.78	++++	++++	++++	++++	++++	" " " 21, 23, 26, 28

The organic and inorganic arsenicals, including the diazotate, were all given in maximal tolerated amounts, as determined beforehand by repeated toxicity tests in adult ZBC mice weighing 22 to 28 gm.

Additional materials tested in the two experiments summarized in this table, with outcome similar to that in Groups 1 and 15: 2.0 ml., each of 4 mice, days 8 to 13.

1. Normal horse serum.
2. 4 per cent bovine albumin in 0.9 per cent NaCl solution.
3. 4 per cent bovine gamma globulin in 0.9 per cent NaCl solution.
4. 4 per cent sodium caseinate in 0.9 per cent NaCl solution.
5. 4 per cent ovalbumin (twice crystallized) in 0.9 per cent NaCl solution.

In further experiments, two densely colored conjugates were made by conjugating 4-arsonophenyldiazotate with equal part of 8 per cent Difco peptone at 22°C., pH 7.4, 15 minutes, the conjugates being then precipitated with alcohol. One material contained 2.54 mg. As/ml. and 1.6 mg./non-protein nitrogen/ml.; both materials proved lethal when given in dilution 1:2. Neither material had any effect on established 6C3HED lymphomas in ZBC mice when given in maximal tolerated amounts (dilution 1:2, 2.0 ml. each day) on 6 successive days beginning on day 7. The same was true of a solution containing 80 mg./ml. of yellowish crystals procured from the interaction of 4-arsonophenyldiazotate on histidine (As and N content not determined).

* ++++, palpable lymphomas in both groins of each test animal measuring approximately 1.0 cm. across on day 8 and enlarging progressively thereafter as in the untreated animals of Charts 1 and 2.

GL, signs of generalized lymphomatosis.

?, palpable mass, softer and usually smaller than those in groins of control animals.

0, no palpable tumors, mouse sleek and lively.

6.4 or 7.4, 0°C., for 2 minutes—that is to say, under conditions in which somewhat less arsenic should theoretically have been attached to the proteins than was so when conjugation was carried out for comparable periods at pH 9.5, 0°C.,—were largely devoid of effect upon Lymphoma 6C3HED cells *in vivo*, whereas the untreated serum acted powerfully upon the cells, as in previous studies (2), the findings suggesting that the active protein constituent of the serum had been altered. So, too, an arsenic–azo-immune globulin which had been conjugated with 4-arsonophenyldiazotate, had much less capacity to kill Lymphoma 6C3HED cells *in vitro* in the presence of complement than had globulin separated from the untreated serum.

Since arsenic-azoproteins with powerful activity against Lymphoma 6C3HED cells *in vivo* were regularly procured when any one of the albumins or globulins employed was conjugated in equal parts with the 4-arsonophenyldiazotate—either at pH 9.5, 0°C., for periods of ½ to 2 minutes or at pH 7.4, 22°C., for periods of 5 to 15 minutes or longer—such materials were used empirically throughout the work, and systematic attempts were not made to define precisely the conditions of conjugation that would yield arsenic-azoproteins with maximal activity and minimal toxicity. The materials varied somewhat in optical density, and in content of arsenic and protein, as has been shown. Comparative tests disclosed that they varied also in capacity to act upon Lymphoma 6C3HED cells *in vivo*, and in toxicity for the animal hosts.

For example, a number of arsenic-azoglobulins, separated from mixtures of equal parts of the 4-arsonophenyldiazotate with normal horse serum or normal rabbit serum which had been held at 0°C., pH 9.5, for ½ minute, had optical densities of approximately 0.34 when measured spectrophotometrically at pH 7.4, dilution 1:50, mμ 400. Such preparations regularly induced regression of established 6C3HED lymphomas when given to adult ZBC mice in amounts of 1.0 or 2.0 ml. on 3 to 6 successive days beginning on the 8th or 10th day following the implantation of 0.5 million cells in each groin of the test animals. Under such circumstances the mice usually maintained their respective weights ± 1 or 2 gm., and remained lively and devoid of signs of arsenic toxicity or other illness throughout the course of the injections and thereafter (see Charts 1 and 2 for examples). Preparations made by coupling the diazotate and serum proteins (or with albumins or globulins separated from them) at 0°C. pH 9.5, for periods of 1 to 2 minutes, or at 22°C., pH 7.4, for periods of 5 to 15 minutes, usually had optical densities ranging from 0.42 to 0.52. Such materials almost always induced complete regression of established 6C3HED lymphomas in ZBC mice when given in amounts of 0.5 to 2.0 ml. on 3 to 5 successive days, usually without inducing loss of weight or other signs of toxicity in the animal hosts. Those conjugated for longer periods—*e.g.*, at 0°C., pH 9.5, for 8 minutes, or at 22°C., pH 7.4, for 30 minutes or longer—in addition to being more deeply colored (OD, 0.66 or greater), often induced regression of established 6C3HED lymphomas when given in amounts of 0.5 ml. on successive days. But such materials proved toxic as a rule (inducing marked tremors of the head and extremities, sometimes with cachexia and death) when given in amounts of 1 or 2 ml. on 3 or more successive days to ZBC mice weighing 20 to 24 gm.

Considered together, the findings given thus far make plain that certain arsenic-azoproteins, when suitably prepared and administered, regularly induce regression of established 6C3HED lymphomas in susceptible mice without harming the latter perceptibly, and that no such effect is brought about by the

proteins employed, by simple mixtures of arsanilic acid and the proteins, by sulfur-azoproteins, or by any of a variety of inorganic and organic arsenicals when these are given in maximal tolerated amounts.

Necrosis of Lymphoma 6C3HED Cells in ZBC Mice Treated with Arsenic-Azoproteins

Lymphoma 6C3HED cells implanted in the subcutaneous tissues of untreated ZBC mice usually begin to proliferate within 24 to 36 hours, as numerous studies have shown—the proliferating cells forming palpable tumor masses within 5 to 7 days which enlarge progressively until death of the animals, usually with such signs of generalized lymphomatosis as labored respiration, subcutaneous edema, and palpably enlarged liver and spleen. Postmortem examinations with histological studies have shown that the proliferating lymphoma cells find their way promptly to the lymph nodes of groins and axillae, and that they can often be found in massive numbers in the liver, spleen, kidneys, and other viscera of the hosts in the later stages of the process. When the subcutaneous lymphomas have become 2.5 to 3.5 cm. across and quite turgid—that is to say, during the period 16 to 26 days following implantation of the cells—they often press upon the overlying skin to the extent that the cells comprising this become necrotic; within such growths, as also just under the tautly stretched skin, sheets and masses of necrotic lymphoma cells can often be found. One may suppose that the lymphoma cells die in such circumstances in consequence of turgidity or anoxia, for numerous histologic studies have shown that necrotic cells are rarely to be found in the rapidly enlarging growths—measuring 1.0 to 2.0 cm. across—during the period 7 to 14 days following implantation (Fig. 1). By contrast, numerous observations have shown that proliferating Lymphoma 6C3HED cells in the groins of ZBC mice that had been treated daily with suitable arsenic-azoproteins beginning 8 to 11 days following implantation, promply begin to die as individuals throughout the tumor masses, and often in proximity to dilated blood vessels, the process of necrosis continuing rapidly until no viable lymphoma cell is left. The non-neoplastic cells of the host animals, by contrast, and notably those of lymph nodes, thymus, and bone marrow, remained morphologically unchanged. Figs. 2, 3, and 4 illustrate the findings.

Susceptibility of Lymphoma 6C3HED Cells to Effects of Various "Anti-Lymphoma" Agents in Vivo: Tests for Effects of Arsenic-Azoproteins on Lymphoma Cells of Other Types

In several tests, about which more will be said further on, arsenic-azoproteins that proved lethal for Lymphoma 6C3HED cells *in vivo* had little or no ability to stay the growth of Lymphoma AKRL1 or Lymphoma L4946 cells in AKR/Jax mice, or that of L1210 cells in D2BC mice, even when the conjugates were

given on the day of implantation and on 4 successive days thereafter in maximal tolerated amounts. Since normal guinea pig serum, when suitably administered, regularly brings about the regression of Lymphoma 6C3HED cells *in vivo* (2) but is devoid of effect on the cells of numerous other mouse lymphomas including those of AKRL1, L4946, and L1210, it seemed reasonable to suppose that the effects of the arsenic-azoproteins and those of normal guinea pig serum might be related, both being dependent perhaps upon some special liability of Lymphoma 6C3HED cells; and also that Lymphoma 6C3HED cells might be specially vulnerable to the effects of such agents as are capable of acting against lymphocytic cells *in vivo*. Experimentation however, has proved the suppositions fallacious.

Any attempt to answer satisfactorily the question whether Lymphoma 6C3HED cells are specially vulnerable to such agents as might act *in vivo* upon neoplastic lymphoma cells, or on non-neoplastic lymphocytic cells generally, is handicapped initially by the fact, now widely recognized, that the "chemotherapeutic" agents thus far described in the scientific literature have at best only feeble and uncertain effects against the cells of mouse lymphomas *in vivo* (1, 8–14). Even so it seemed essential to find out whether Lymphoma 6C3HED cells are specially susceptible to the effects of such agents as are now available. Hence a number of tests were made for effects on these cells *in vivo* of the following materials, each given on 6 successive days in maximal tolerated amounts: amethopterin, chlorambucil, 6-mercaptopurine, 8-azaguanine, ThioTEPA, azaserine, 6-azauracil, DON, and 5-fluorouracil.[2] In extensive trials, none of these chemical compounds had any such effect, though when given in toxic but sublethal amounts, both DON and 5-fluorouracil brought about a transitory softening of subcutaneous lymphomas in the test mice and some slight prolongation of host survival time. The findings recorded in Table II are representative of a considerably larger experience in attempts to influence established 6C3HED lymphomas in ZBC mice with these compounds. It can be added that in several other experiments none of these materials prevented the development of palpable 6C3HED lymphomas and generalized lymphomatosis in ZBC mice when each was given in maximal tolerated amounts to a group of 4 implanted mice on the day of implantation and on each of the succeeding 4 days—under conditions, that is to say, which were propitious for the action of the chemical compound and unfavorable for the survival of the lymphoma cells; this has also proved true of cortisone.

By contrast, adequate experimental materials were at hand for resolving the question whether arsenic-azoproteins and normal guinea pig serum exert their effects on Lymphoma 6C3HED cells *in vivo* through some related means. For by undertreating C3H/Jax mice carrying established 6C3HED lymphomas, Dr. Nelson Holmquist and I have recently produced two sublines of Lymphoma 6C3HED cells which, although identical with the original line of 6C3HED cells in morphology and in growth characteristics upon transplantation in susceptible mice, have proved totally refractory to the effects of guinea pig serum during more than 20 serial transplantations. Both of the serum-resistant sublines are susceptible to the effects of arsenic-azoproteins, as several experiments have shown. Table III gives the findings of a representative experiment.

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[2] Acting for the original sources, Dr. Chester Stock of The Sloan-Kettering Institute generously supplied the chemical compounds. Throughout the paper, the terms DON and thioTEPA have been used to designate 6-diazo-5-oxo-norleucine and N, N′, N″, triethyleneimino thiophosphoramide, respectively.

Mention has already been made of the fact, clearly shown in several experiments and documented in Table IV, *q.v.*, that arsenic-azoprotein preparations—each capable of acting powerfully upon Lymphoma 6C3HED cells, as was shown in subsidiary tests—had relatively little or no effect on the cells of Lymphomas AKRL1 and L4946 in AKR mice, or upon those

TABLE II

Tests for Effects of Various "Chemotherapeutic" Agents on Lymphoma 6 C3HED Cells in Vivo

Experimental groups 4 ZBC mice (22–28 gm.) in each group—all implanted with 0.5 million 6C3HED cells in each groin, day 1	**Outcome** Growths in 4 test mice					
Intraperitoneal injections: 2.0 ml. each animal, days 8 to 13	Days following implantation: 8	12	15	18	22	Subsequent course
1. Nil—untreated controls	++++	++++	++++	++++	++++	GL-died—days 23, 23, 26, 27
2. 4-arsonophenylazo—bovine albumin, OD—0.53	++++	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0	All survived—60 days
3. Amethopterin, 0.08 mg./ml.	++++	++++	++++	++++	++++	GL-died—days 23, 27, 27, 31
4. " 0.04 " "	++++	++++	++++	++++	++++	GL- " " 25, 29, 29, 29
5. " 0.02 " "	++++	++++	++++	++++	++++	GL- " " 23, 23, 28, 28
6. " 0.01 " "	++++	++++	++++	++++	++++	GL- " " 21, 25, 28, 28
7. Chlorambucil, 0.16 mg./ml.	++++	++++	++++	++++	++++	GL-died—days 25, 25, 27, 27
8. 6-Mercaptopurine, 0.03 mg./ml.	++++	++++	++	++	++	GL-died—days 12, 14, 30, 31
9. 8-Azaguanine 0.75 mg./ml.	++++	++++	++++	+++	+++	GL-died—days 15, 25, 27, 29
10. ThioTEPA, 0.04 mg./ml.	++++	++++	++++	++++	++++	GL-died—days 23, 23, 23, 23
11. Azaserine 0.20 mg./ml.	++++	++++	++++	+++	+++	GL-died—days 14, 28, 29, 30
12. " 0.10 " "	++++	++++	++++	++++	++++	GL- " " 25, 26, 30, 31
13. 6-Azauracil, 5.0 mg./ml.	++++	++++	++++	+++	+++	GL-died—days 15, 23, 26, 27
14. DON 0.004 mg./ml.	++++	? ? ? ?	? ? ? ?	+++	+++	GL-died—days 17, 27, 31, 35
15. " 0.002 " "	++++	++++	? ? ++	++++	++++	GL- " " 26, 31, 31, 32
16. " 0.001 " "	++++	++++	++++	++++	++++	GL- " " 27, 29, 29, 30
17. 5-Fluorouracil, 0.5 mg./ml.	++++	++++	? ? ? ?	++++	++++	GL-died—days 30, 31, 33, 35
18. " 0.25 " "	++++	++++	++++	++++	++++	GL- " " 27, 27, 29, 29

Each material was given in maximal tolerated amounts as determined beforehand on 22 to 28 gm. ZBC mice.
++++, growths in both groins of each test mouse, approximately 1.0 cm. across on day 8, enlarging progressively thereafter as in the untreated control animals.
GL, signs of generalized lymphomatosis.
?, palpable mass, softer and usually smaller than those in groins of control animals.
0, no palpable tumors, mouse sleek and lively.

of Lymphoma L1210 in D2BC or DBA mice. The effects of arsenic-azoproteins on the cells of Lymphoma E9514—a transplanted growth that originated in a C3H/Jax mouse in the laboratory of Dr. George Snell of The Jackson Memorial Laboratories, Bar Harbor, and the only other lymphoma against which the arsenic-azoproteins have been employed thus far—have interest in this and other relations. For although guinea pig serum has no effect on the cells of Lymphoma E9514 *in vivo*, as many tests have shown (1), the arsenic-azoproteins act power-

TABLE III

Effects of Arsenic-Azoproteins on Sublines of 6C3HED Cells that are Resistant to the Effects of Guinea Pig Serum in Vivo

Experimental groups	Growths in four test mice of each group				
I. 4 ZBC mice in each group—all implanted in each groin with 0.2 million 6C3HED cells of original line; given intraperitoneal injections as indicated	Days: 8	11	15	21	Subsequent course
1. Untreated controls	++++	++++	++++	++++	GL-died—days 23, 24, 26, 28
2. Given normal guinea pig serum, 2 ml. each animal, days 8 to 10	++++	0 0 0 0	0 0 0 0	0 0 0 0	All survived—60 days
3. Given 1.0 mg. arsanilic acid in 2 ml. normal horse globulin, each animal, days 8 to 10	++++	++++	++++	++++	GL-died—days 24, 24, 26, 28
4. Given 4-arsenophenylazo-horse globulin (OD = 0.34) 2 ml., each animal, days 8 to 10	++++	0 0 0 0	0 0 0 0	0 0 0 0	All survived—60 days
5. Given 4-arsenophenylazo-horse globulin (OD = 0.46), 1 ml., each animal, days 8 to 10.	++++	0 0 ++	0 0 0 0	0 0 0 +	Three mice survived 60 days; one, GL-died—day 32
II. 4 ZBC mice in each group—all implanted in each groin with 0.2 million 6C3HED cells of resistant subline 1RG; given intraperitoneal injections as indicated	Days: 8	11	15	21	Subsequent course
1. Untreated controls	++++	++++	++++	++++	GL-died-days 23, 28, 29, 32
2. Given normal guinea pig serum, 2 ml., each animal, days 8 to 10.	++++	++++	++++	++++	GL-died-days 24, 26, 28, 32
3. Given 1.0 mgm. arsanilic acid in 2 ml. horse globulin, each animal, days 8 to 10	0+++	0 +++	0 +++	0 +++	One mouse survived 60 days. Other three: GL-died—days 26, 28, 32
4. Given 4-arsonophenylazo-horse globulin (OD = 0.34) 2 ml., each animal, days 8 to 10	++++	0 0 0 0	0 0 0 0	0 0 0 0	All survived—60 days
5. Given 4-arsonophenylazo-horse globulin (OD = 0.46) 1 ml., each animal, days 8 to 10	++++	0 0 0 0	0 0 0 0	0 0 0 0	All survived—60 days
III. 4ZBC mice in each group—all implanted in each groin with 0.2 million 6C3HED cells of resistant subline 2 RG; given intraperitoneal injections as indicated	Days: 8	11	15	21	Subsequent course
1. Untreated controls	++++	++++	++++	++++	GL-died-days 26, 28, 28, 29
2. Given normal guinea pig serum—2 ml., each animal, days 8 to 10	++++	++++	++++	++++	GL-died-days 24, 25, 26, 28
3. Given 1.0 mg. arsanilic acid in 2 ml. horse globulin, each animal, days 8 to 10	++++	++++	++++	++++	GL-died-days 24, 25, 25, 28
4. Given 4-arsonophenylazo-horse globulin (OD = 0.34) 2 ml. each animal, days 8 to 10	++++	0 0 0 0	0 0 0 0	0 0 0 0	All survived—60 days
5. Given 4-arsonophenylazo-horse globulin (OD = 0.46) 2 ml., each animal, days 8 to 10	++++	0 0 0 0	0 0 0 0	0 0 0 0	All survived—60 days

++++, growths in both groins of each test mouse, approximately 1.0 cm. across on day 8, enlarging progressively thereafter as in the untreated control animals.

GL, signs of generalized lymphomatosis.

0, no palpable tumors, mouse sleek and lively.

fully upon them, as is shown in one of the experiments summarized in Table IV. In this test, it can be seen that C3H/Jax mice, implanted with E9514 cells and treated with one or the other of two arsenic-azoprotein preparations on the day of implantation and on each of the succeeding 2 days, all failed to develop palpable lymphomas and remained lively, without signs of lymphomatosis, throughout an observation period of 42 days, while untreated control animals without exception developed palpable subcutaneous lymphomas and died with signs of generalized lymphomatosis during the period 15 to 20 days. This result was substantiated in a subsequent experiment. Furthermore in four additional experiments fully established

TABLE IV

Tests for Effects of Arsenic-azoproteins on Cells of Lymphomas E9514, L1210, and L4946 in Vivo

Experiments and experimental groups	Growths in four test mice of each group				
I. 4 C3H/Jax mice in each group—all implanted with 0.5 million E9514 cells in each groin. Each animal given 2 ml. on days 1, 2, 3:	Days following implantation: 8	11	14	18	Subsequent course
1. Nil—untreated controls	++++	++++	++++	++++	GL-died—days 15, 20
2. 4-arsonophenylazo-horse globulin, OD—0.38	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0	All survived (42 days)
3. 4-arsonophenyl-bovine albumin, OD—0.26	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0	" " "
II. 4 AKR/Jax mice in each group—all implanted with 0.06 million L4946 cells in each groin. Each animal given 2 ml., days 1, 2, 3, 4, 5:	Days following implantation: 9	11	14		Subsequent course
1. Nil—untreated controls	++++	++++	++++		GL-died—days 16, 16, 16 17
2. 4-arsonophenylazo-horse globulin OD—0.38	++++	++++	++++		" " " 16, 16, 16, 17
3. 4-arsonophenylazo-horse globulin OD—0.46	++++	++++	++++		" " " 16, 16, 16, 17
4. 4-arsonophenylazo-bovine albumin OD—0.26	++++	++++	++++		" " " 16, 16, 16, 17
5. 4-arsonophenylazo-bovine albumin OD—0.42	++++	++++	++++		" " " 16, 16, 16, 16
III. 4D2BC mice in each group—all implanted with 0.02 million L1210 cells in each groin. Each animal given 2 ml., days 1, 2, 3, 4, 5:	Days following implantation: 11	14	16		Subsequent course
1. Nil—untreated controls	0 0++	++++	++++		GL-died—days 16, 17, 17, 21
2. 4-arsonophenylazo-horse globulin OD—0.38	0 0++	? ?++	++++		" " " 18, 19, 21, 21
3. 4-arsonophenylazo-horse globulin OD—0.46	0 0++	? ?++	++++		" " " 19, 19, 19, 21
4. 4-arsonophenylazo-bovine albumin OD—0.26	0 0++	? ?++	++++		" " " 18, 19, 21, 21
5. 4-arsonophenylazo-bovine albumin OD—0.42	0 0++	? ?++	++++		" " " 19, 20, 21, 21

++++, growths in both groins of each test mouse, approximately 1.0 cm. across on day 8 enlarging progressively thereafter as in the untreated control animals.
GL, signs of generalized lymphomatosis.
0, no palpable tumors, mouse sleek and lively.

subcutaneous E9514 lymphomas (which measured 0.8 to 1.2 mm across on the 8th day following implantation) promptly dwindled and disappeared when one or another of several arsenic-azoproteins was injected intraperitoneally on 3 to 5 successive days beginning on day 8 in each of 16 mice. Within a few days, however, all the treated mice manifested palpably enlarged livers and spleens, and they all soon sickened and died, usually within a day or 2 following death of the untreated controls, histologic studies showing that the groins of the treated animals were devoid of Lymphoma E9514 cells while their livers and spleens were massively invaded.

It remains to be learned why the arsenic-azoproteins should fail to stay the growth of Lymphoma E9514 cells in livers and spleens of susceptible mice while overcoming them in subcutaneous tissues; and also why the conjugates, while capable of acting powerfully upon Lymphoma 6C3HED cells *in vivo*, should prove devoid of effect upon the cells of Lymphomas AKRL1, L1210, and L4946.

DISCUSSION

Only those conjugates containing arsenic in combination with relatively large amounts of protein were notably effective in overcoming Lymphoma 6C3HED cells *in vivo* (Table I). Taken together with the other findings of the work, the fact suggests that the arsenic, when combined with protein in this way, acts directly and presumably to some extent selectively on the proliferating lymphoma cells, though precisely why this should be so remains unknown. For, although much is known about the pharmacologic and toxic effects of arsenic upon mammalian organisms (15), and much also about its selective effects upon spirochetes and trypanosomes (16), relatively little is known of its effects upon mammalian cells as such, though in this relation, Forkner some time ago employed potassium arsenite extensively in the treatment of chronic myelogenous leukemia in human beings (17, 18), while Dustin has shown that sodium cacodylate can arrest division and produce nuclear abnormalities in mouse sarcoma cells (19), and Fell and Allsopp have noted that lewisite is highly toxic to cultured embryonic cells (20), perhaps in part because of its action upon sulfhydryl enzyme systems essential for their metabolism (15). In relation to the part played by the protein component of the arsenic-azo conjugates, it may be significant that such conjugates are antigenic, as Landsteiner has shown (6); hence it may be inferred that they enter into close association with living cells. Furthermore, a number of other workers—notably Sabin (21), Madden and Whipple (22), Oliver (23), and Brachet (24)—using diverse experimental materials and methods, have provided evidence that living mammalian cells can take in and metabolize proteins, while Busch and Greene (25), and Kent and Gey (26) have recorded observations indicating that this is true of certain tumor cells as well. Certainly the possibility exists that in the present work the protein component might somehow have brought the conjugated arsenic into close association with lymphoma cells, thus giving rise to the effects here described. To learn through experiment whether this be so is another matter, however, as is also the question whether the bound arsenic induces necrosis of Lymphoma 6C3HED cells *in vivo* by acting upon essential sulfhydryl enzymes.

Whatever the means may be whereby arsenic-azoproteins bring about necrosis of Lymphoma 6C3HED cells *in vivo*, it is obvious that the conjugates failed under more or less comparable circumstances to act conspicuously on mouse lymphoma cells of several other types. For although the arsenic-azoproteins readily overcame Lymphoma E9514 cells growing in the subcutaneous

tissues of susceptible mice, they had little or no effect once these cells had reached the livers and spleens of susceptible hosts, and they were essentially devoid of effect *in vivo* on the cells of Lymphomas AKRL1, L4946, and L1210 (Table IV). In this relation, special interest centers around the fact that Lymphoma 6C3HED cells are highly susceptible *in vivo* to the effects of arsenic-azoproteins and to those of guinea pig serum (1, 2). Since the conjugates are effective against sublines of Lymphoma 6C3HED cells that are resistant to the effects of guinea pig serum (Table III), it is clear that the arsenic-azoproteins and guinea pig serum probably act through unrelated means. Furthermore, since Lymphoma 6C3HED cells are largely uninfluenced *in vivo* by amethopterin, chlorambucil, 6-mercaptopurine, 8-azaguanine, azaserine, 6-azauracil, 5-fluorouracil, ThioTEPA, and DON (Table II), it seems plain that they are not specially liable to the effects of such "chemotherapeutic" compounds as have been widely presumed to act upon lymphoma cells generally. The findings deserve consideration in relation to the results of numerous attempts made heretofore to overcome proliferating lymphoma cells in susceptible mice under controlled experimental conditions.

Much work by others has shown that certain chemical compounds, although often proving toxic for the animal hosts in the amounts given, have exerted effects—at best only feeble and uncertain—upon a few types of lymphoma cells *in vivo* while being devoid of noteworthy effect on lymphoma cells of other types, as is now widely recognized (1, 8–14). So, too, immune serums, prepared in rabbits with mouse lymphoma cells as antigens, share these limitations, though in lesser degree; for while such serums have acted upon all the mouse lymphoma cells thus far exposed to their effects *in vivo*, they have proved considerably more effective against the cells of some lymphomas than against those of others, and none has been sufficiently powerful to cure susceptible mice with advanced lymphomatosis (1). By contrast, guinea pig serum, when suitably prepared and administered, regularly cures susceptible mice with advanced 6C3HED lymphomatosis, its effects in this relation being far more powerful than are those of any other antilymphoma agent thus far described in the scientific literature (1, 2, 27); yet it has proved devoid of effect upon all but two of the 14 types of mouse lymphoma cells tested thus far (1).

From the foregoing, at least two inferences can be drawn: (*a*) that lymphoma cells of a single type may be highly susceptible to the effects of diverse "anti-lymphoma" agents (which act in different ways upon them) and at the same time prove refractory to the effects of other "anti-lymphoma" agents, and (*b*) that wide differences exist between various types of mouse lymphoma cells that resemble one another quite closely in growth characteristics following transplantation in susceptible mice and in morphologic characters as determined by ordinary means.[3] More refined methods than those hitherto employed

[3] In this relation, Shrek, Friedman, and Leithold have recently provided evidence that neoplastic lymphocytes of human beings differ widely in sensitivity to the effects of x-rays and chemotherapeutic agents *in vitro* (*J. Nat. Cancer Inst.*, 1958, **20**, 1037).

will obviously be required to define precisely the means whereby anti-lymphoma agents exert their effects upon lymphoma cells *in vivo*, and the nature of the intrinsic differences between lymphoma cells of various types. In these relations, it remains to be learned whether other metallo-protein compounds will act upon lymphoma cells *in vivo* and thus perhaps disclose more about their nature, and also whether a cytotoxic chemical can be attached to anti-lymphoma immune globulins in such a way as to enhance their effects on lymphoma cells *in vivo*.

SUMMARY

Conjugates made by coupling diazotized arsanilic acid with one or another of a variety of proteins regularly brought about the complete regression of established 6C3HED lymphomas in living mice without perceptibly harming the latter, while untreated control animals regularly died with lymphomatosis. Histologic studies made plain that the lymphoma cells promptly die in mice treated with the arsenic-azoproteins, while those in untreated control animals continue to proliferate. Various inorganic and organic arsenicals (including arsanilic acid and 4-arsonophenyldiazotate) were essentially devoid of effect on the lymphoma cells *in vivo*, and this proved true as well of the proteins employed (serum albumins and globulins procured from several species, casein, and ovalbumin). Mixtures of arsanilic acid and the several proteins, various sulfur-azoproteins, and a number of other substances—*viz.*, amethopterin, chlorambucil, 6-mercaptopurine, 8-azaguanine, azaserine, 6-azauracil, 5-fluorouracil, thioTEPA, and DON, each given in maximal tolerated amounts—also failed to influence notably the course of established 6C3HED lymphomas *in vivo*.

Although readily overcoming Lymphoma E9514 cells growing in the subcutaneous tissues of susceptible mice, the arsenic-azoproteins had little or no effect once these cells had reached the livers and spleens of susceptible hosts. Furthermore the arsenic-azoproteins had little or no effect *in vivo* on the cells of Lymphoma AKRL1, L1210, and L4946.

The findings were considered in relationship to the respective susceptibilities of several types of lymphoma cells to other anti-lymphoma agents—notably guinea pig serum, immune serums prepared in rabbits with mouse lymphoma cells as antigens, and a variety of chemical compounds. Taken together, the observations provide proof that lymphoma cells of various types, although resembling one another quite closely in growth characteristics following transplantation in susceptible hosts, and in morphology as disclosed by ordinary microscopy, differ notably in susceptibility to the effects of the several anti-lymphoma agents.

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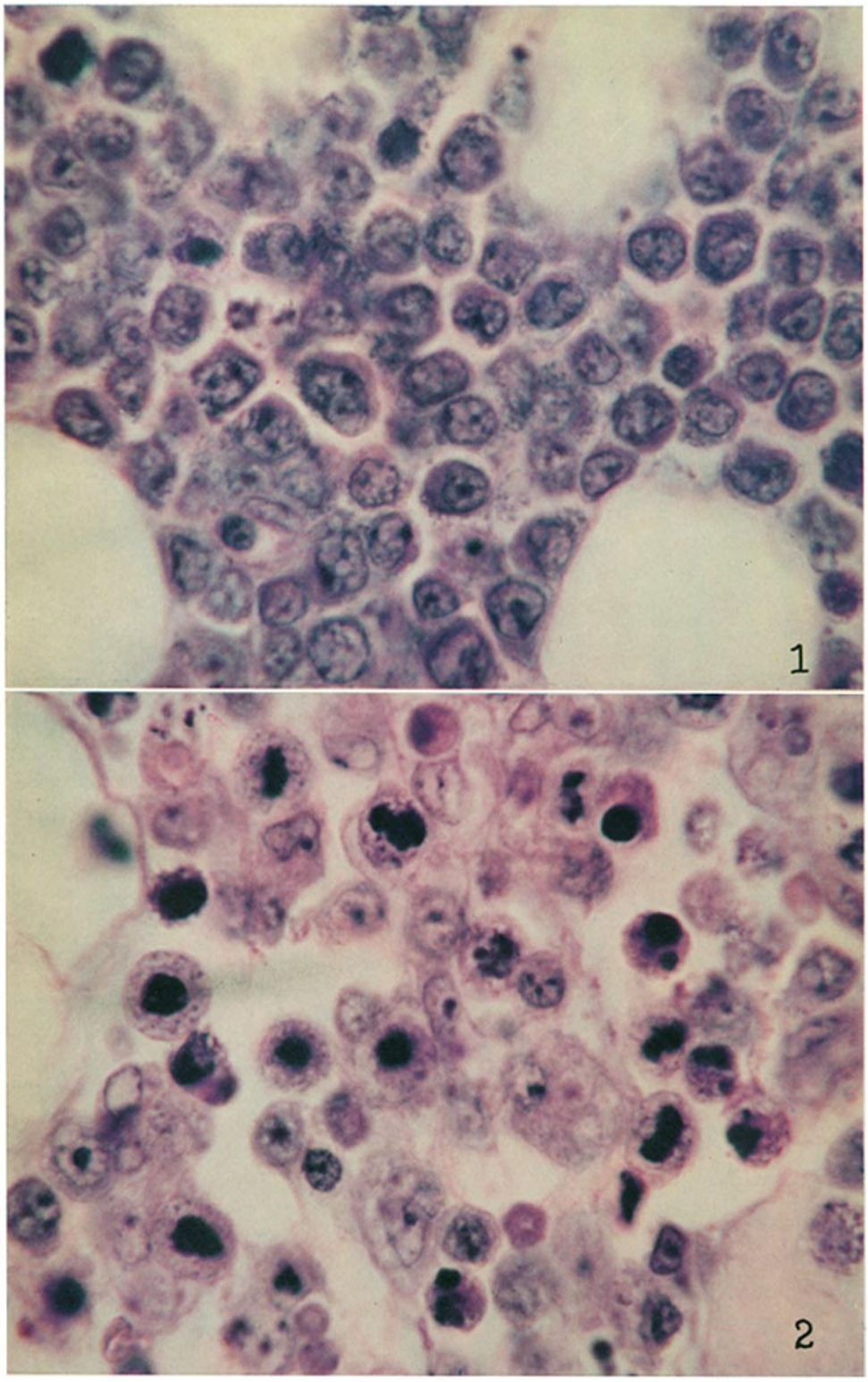
EXPLANATION OF PLATES

PLATE 44

The photographs were made by Mr. Julius Mesiar and Dr. Harry Browne. The sections were stained with methylene blue and eosin.

FIG. 1. Lymphoma 6C3HED cells proliferating in the subcutaneous areolar tissue of a ZBC mouse on the 13th day following implantation of 0.5 million lymphoma cells in each groin. Four additional untreated mice implanted at the same time all had progressively enlarging lymphomas and died respectively on days 24, 26, 28, 29. Initially × 800; enlarged to approximately × 1400.

FIG. 2. Necrotic and degenerating Lymphoma 6C3HED cells in the groin of a ZBC mouse that had been treated with arsenic–azo horse globulin. 0.5 million lymphoma cells had been implanted in each groin on day 1—same cell suspension as used in the animal of Fig. 1; on day 8, tumors measuring 1.2 and 1.3 cm. across were present in left and right groins; on days 10 to 12, 2 ml. arsenic–azo horse globulin (OD: 0.46) were given intraperitoneally. Tumors procured for histologic study on day 13. Permanent regression of lymphomas in 4 additional animals given the same arsenic–azoprotein preparation on days 10 to 14—2 ml., each mouse, each day. Initially × 800; enlarged to approximately × 1400.

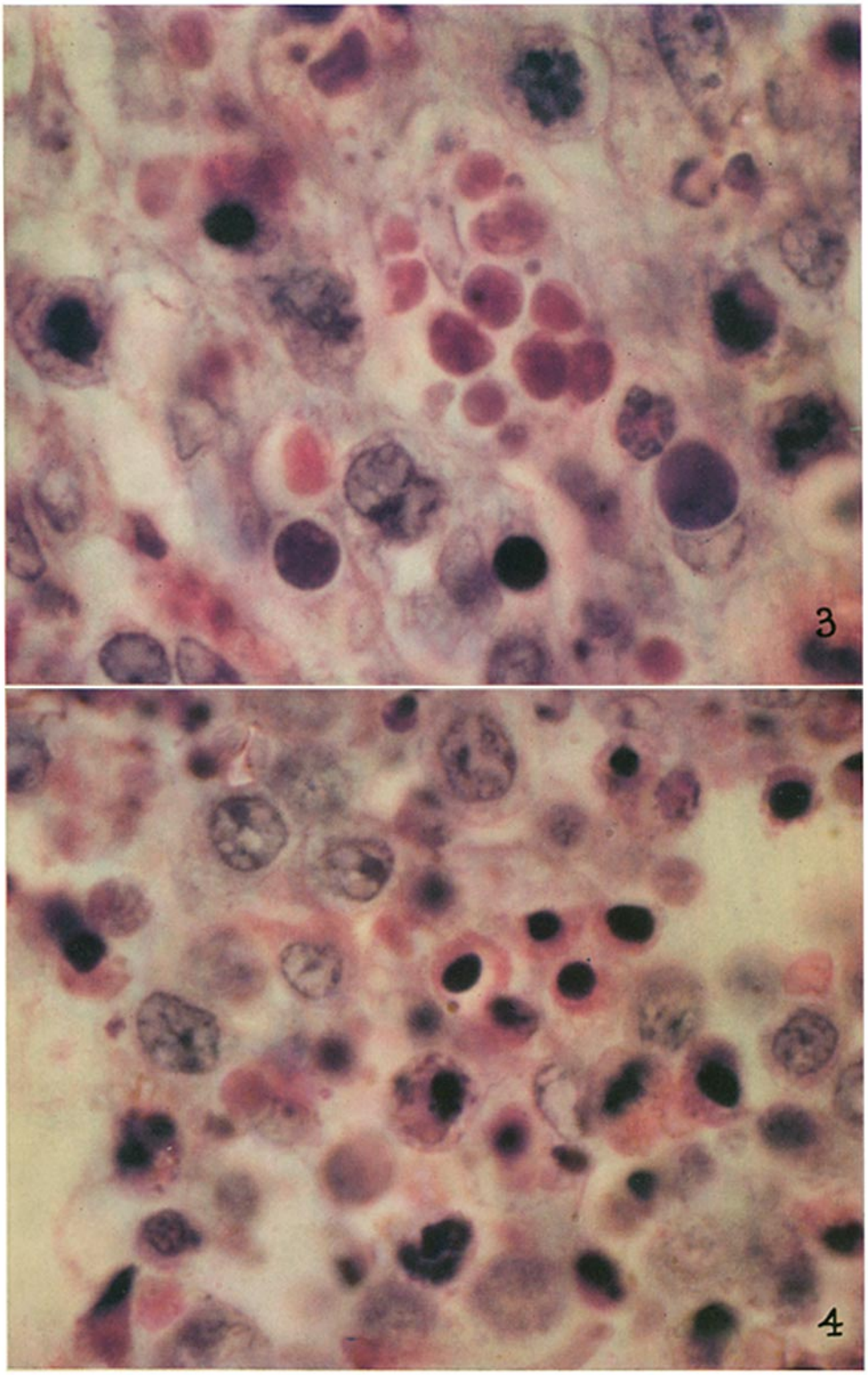


(Kidd: Arsenic-azoproteins and lymphoma cells)

PLATE 45

FIG. 3. Necrotic lymphoma cells in the groin of a second treated mouse of the same experiment. 2 ml. arsenic–azo bovine albumin, OD: 0.36, given on days 10 and 11; tumors procured for histologic study on day 12. This arsenic–azo protein preparation also brought about permanent regression of 6C3HED lymphomas in 4 additional ZBC mice each given 2 ml., days 10 to 14. Initially × 1260; enlarged to approximately × 1700.

FIG. 4. Necrotic lymphoma cells in the growth of the opposite groin of treated mouse of Fig. 1. Initially × 1260; enlarged to approximately × 1700.



(Kidd: Arsenic-azoproteins and lymphoma cells)