Modifications of Phagocitary Activity by Splenic Cells of Rat Carriers of Sarcoma, Isolated *in vitro*

Following upon observations made during previous work[1-3], I have observed that rat carriers of big Galliera sarcoma show a considerable increase of spleen, reaching sometimes up to five times the bulk of the spleen of normal rats.

Analogous observations have been made by others[4-6] on animal carriers of tumors, and WINN demonstrated that spleen of animal carriers of tumors produces a cellular antibody that is specific for the tumor. Histological modifications of the spleen of rat carriers of Galliera sarcoma consist in hyperplasia and hypertrophy of the reticulo-endothelium, whose cells sometimes show phagocitated granules and sometimes vacuoles in cytoplasm; thickening of the connective tissue of the spleen was also observed.

On the basis of such observations, possible functional modifications of the cells of the spleen of rat carriers of Galliera sarcoma were investigated studying the phagocitary activity of the splenic cells isolated *in vitro*, according to the technique described by SHEPARD[7-9] and duly modified.

Material and Method. I used for this research 30 rats having an average weight of about 230 g each, divided into two groups. The first (control) group consisted of 15 normal rats; the second group of 15 rats, carriers of big non-ulcered sarcoma. After having killed each animal by beheading and further bleeding, I took away the spleen, whose total weight was controlled. Furthermore I homogenized 0.5 g of splenic tissue in 10 ml of sterile physiological solution of NaCl; I then put the homogenates in sterile test-tubes, for a spontaneous sedimentation of 5 min, in order to remove the non-homogenated tissue. The supernatant was centrifugated for 90 sec at 300 *g*, in order to collect only splenic cells, without erythrocytes; the cells were suspended in 10 ml of sterile physiological solution and centrifugated a second time at the same speed for 90 sec. The pureness of the cells isolated *in vitro* was controlled by microscope. The splenic cells were then suspended in 2 ml of sterile physiological solution in order to study their phagocitic activity. In a sterile test-tube I put 0.5 ml of suspension of splenic cells with 0.5 ml of fresh guinea pig complement and with 7 ml of a fresh suspension of *Staphylococcus pyogenes aureus*, Oxford stock; after mixing, I put the test-tube in a thermostat at + 37° C for 3 h, having found in the preliminary tests that such period of incubation was the optimal.

Furthermore I made slides of the cells, which were fixed by heat and stained by Gram's method. For each experience I counted the number of cells having phagocited one or more staphylococchi, on a total of 400 cells, and calculated the average percentage for each experiment.

	Controls	Rat carriers of Galliera sarcoma
	mg	mg
Average weight of spleen	926 ± 45	3369 ± 1316
Phagocitic activity	4.1% ± 0.9	11.2% ± 1.8

The Table refers to the average data of the total weights of the spleens and of the phagocitic activity of the splenic cells of controls and of rat carriers of sarcoma. As regards the dry weights of spleens, I found no difference between the normal rats and the rat carriers of sarcoma.

Conclusion. The results of this research permit the conclusion that the phagocitic activity of the splenic cells of rat carriers of Galliera sarcoma, isolated *in vitro*, is much greater than the phagocitic activity of the splenic cells of normal rat, as observed histologically.

The hypothesis that appears the most probable to explain the increase of the spleen and the increase of phagocitic activity of splenic cells, is that the tumor produce one or more substances (probably also antigens) stimulating the reticulo-endothelial tissue and connective tissue, of which the considerable increase of bulk may be considered responsible.

Riassunto. L'autore ha osservato che l'attività fagocitaria delle cellule spleniche di ratto portatore di sarcoma Galliera, isolate *in vitro*, risulta sensibilmente aumentata rispetto a quella delle cellule di milza di ratto sano. La significatività dei risultati viene accertata calcolando la '*t*' di Fisher.

A. ZINNARI

Istituto di Patologia Generale dell'Università di Genova (Italy), October 16, 1961.

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Macromolecular Hypertension: Hypertensive Cardiovascular Disease from Subcutaneously Administered Polyvinyl Alcohol[1]

It has recently been reported from this laboratory that rats, treated subcutaneously with methyl cellulose, develop ascites, edema, hypertension, foam cells within glomeruli, glomerulonephritis, and a variety of arterial and arteriolar lesions[2,3]. The syndrome may be dependent upon impairment of glomerular circulation caused by accumulation of methyl cellulose within glomerular endothelial and epithelial cells. Such intraglomerular sequestration of methyl cellulose has been described by HUEPER[4,5] and others[6,7], although these investigators did not report the induction of hypertensive cardiovascular disease thereby.

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HUEPER[8,9] also noted that the subcutaneous administration of a 5% solution of polyvinyl alcohol (PVA) into various kinds of animal caused glomerular changes very similar to those caused by methyl cellulose. No mention, however, was made of blood pressure alterations under such circumstances. It seemed desirable to determine whether the macromolecular substances polyvinyl alcohol and methyl cellulose, which have rather similar glomerular effects, would also share the property of inducing hypertensive cardiovascular disease in rats. Accordingly the following experiment was undertaken.

Materials and Methods. Twenty female rats of the Holtzman strain, weighing 75–95 g were divided into two groups. Group 1 consisted of twelve animals given subcutaneously 1 cm^3 per day of 5% polyvinyl alcohol made up in physiological (0.9%) sodium chloride. Eight animals, group 2, received the same volume of saline. Both groups received 1% NaCl solution to drink – a procedure which enhances the development of certain forms of experimental hypertension – and Purina Laboratory Chow. Blood pressures were taken on unanesthetized animals on the first and twenty fifth day of the experiment by a tail plethysmograph, intermediate determinations were made impossible by disruption of services caused by the hurricane 'Carla'. Pressures greater than 150 mm Hg were regarded as hypertensive. At this time the hematocrit, hemoglobin and red cell counts were made from a sample of drawn tail blood and the urine was tested for protein by a reagent impregnated paper method[10]. The animals were killed with ether on the 27th day.

Results. All animals receiving PVA were severely hypertensive by day 25. Systolic pressures ranged 168–232 mm Hg, with a mean of 208 mm Hg. Pressures among controls ranged 120–136 mm Hg with a mean of 126 mm Hg PVA-treated animals also had lower hematocrits, hemoglobin and red cell counts than did controls. One animal which died on the 26th day and another which was killed then because of severe illness, had a particularly severe anemia, and were found at autopsy to have ascites. Estimation of urinary protein indicated that while controls were excreting 0–30 mg% protein, PVA-treated animals were excreting 300–1000 mg%, a considerable proteinuria.

At autopsy PVA-treated rats were found to have kidneys which were grossly enlarged and showed patchy whitish lesions. In one or two animals they were yellow and granular. The hearts were greatly hypertrophied, and in many instances epicardial scars could be detected. There was no abnormality visible in any of the other organs at autopsy, nor in those of controls. Organs were fixed in neutral formalin for subsequent weight and histology.

Treated rats exhibited enlargement of the liver ($P < 0.001$), kidneys ($P < 0.001$), heart ($P < 0.001$) and spleen ($P < 0.02$), slight atrophy of the thymus $P < 0.05$), but no significant adrenal hypertrophy. The data are incorporated in the Table.

Discussion. It is evident that polyvinyl alcohol shares with methyl cellulose the ability to cause hypertensive cardiovascular disease in the rat. Both are carbohydrate emulsion and film forming substances, both are chemically inert and poorly metabolized by or excreted from the body, both are sequestered by the recticuloendothelial system and retained by the glomerulus. The last attribute suggests a possible mode of action.

Previous investigators have attempted to occlude glomerular circulation by injecting particulate matter intravenously, and thereby cause renal ischemia and hypertension. This has not been particularly successful. It is suggested that the use of substances such as PVA and methyl cellulose, which form emulsion in plasma, arrive at the glomeruli and there either occlude capillaries when plasma water is filtered, or are taken up by endothelial cells which, by then swelling, cause glomerular ischemia, may be the agents of choice for this purpose.

It is probable that these macromolecules induce hypertension by a renal mechanism, but if so whether this is due entirely to mechanical interference with intraglomerular circulation or to a more complex mode of action remains to be elucidated. To describe the hemodynamic disturbance induced by such agents the term 'macromolecular hypertension' is suggested. Although not all macromolecules cause hypertension, it is probable that others that do so will be identified. Such hypertension can then be conceptualized as distinct from such forms as, for example, that induced by surgical procedures involving the kidney or by the administration of steroids. Only certain forms of kidney damage will produce 'renal hypertension' and not all steroids produce 'steroid hypertension': nonetheless the terms are widely used.

The pathologic effects of PVA will be described in a subsequent communication, although others have reported on this aspect[8,9]. Renal enlargement is probably largely caused by glomerular retention of the plastic, and changes secondary thereto. The induces glomerular damage probably accounts for the observed proteinuria. Cardiac hypertrophy undoubtedly is secondary to the induced hypertension. Splenomegaly and hepatomegaly result from the uptake of the macromolecule by elements of the reticuloendothelial system. The hematologic findings are in accord with those reported by others. The erythropenia appears to be largely due to sequestration of erythrocytes within the lung, liver and spleen, to osmotic hydremia and to depressed erythropoiesis, but this is said not to account for the hemoglobinemia[11].

Although a high NaCl diet was used in the present experiment, the degree to which the pathophysiological findings depend upon such supplementation has not been established.

Effects of subcutaneous polyvinyl alcohol in the rat

			PVA-Treated	Controls
No. of rats	initial		12	8
	final		11	8
Body weight in g	initial		83 ± 2	89 ± 2
	final		169 ± 6	177 ± 5
Blood Pressure	day 1		119 ± 2	121 ± 2
(mm Hg)	day 25		208 ± 6	126 ± 2
Hemogram	RBC millions/mm^3		5.55 ± 0.32	6.79 ± 0.23
	hematocrit		41 ± 2	47 ± 1
	hemoglobin g%		13.12 ± 0.65	15.26 ± 0.38
Organ weights	adrenals	(mg/100 g)	34.8 ± 1.3	36.2 ± 1.6
	thymus	(mg/100 g)	170 ± 22	241 ± 22
	spleen	(mg/100 g)	427 ± 26	327 ± 24
	heart	(mg/100 g)	474 ± 19	344 ± 8
	kidney	(g/100 g)	2.16 ± 0.19	1.05 ± 0.04
	liver	(g/100 g)	7.15 ± 0.20	5.31 ± 0.12

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Zusammenfassung. Nach subkutaner Injektion von Polyvinylalkohollösung entwickeln Ratten innerhalb eines Monats arteriellen Hochdruck. Gleichzeitig beobachtet man Vergrösserung von Leber, Milz, Herz und Nieren, sowie Thymusatrophie, jedoch keine Nebennierenvergrösserung. Es wird daher vermutet, dass diese Hypertonie, ähnlich der durch Methylcellulose hervorgerufenen, eine Folge von gestörter intraglomerulärer Durchblutung ist.

C. E. HALL and O. HALL

The Carter Physiology Laboratory, University of Texas Medical Branch, Galveston (USA), October 16, 1961.

Der Nachweis praecipitierender Casein-Antikörper im Agar

Für das Aufdecken von Nahrungsmittel-Antikörpern standen uns bis jetzt vier Verfahren zur Verfügung: (1) Die *Praecipitin*-Reaktion, welche mit dem Vorteil der technischen Einfachheit und mit dem Nachteil der geringen Sensibilität behaftet ist. (2) Die *Komplementbindungsreaktion*, die ca. zehnmal empfindlicher als die Praecipitinreaktion ist. Ihr Nachteil liegt in der geringen Spezifität, im Verbrauch von viel Serum, sowie in der umständlichen Technik, die das Ansetzen zahlreicher Kontrollen erfordert. (3) Die Prüfung auf Antikörper durch *Injektion des Antigens in die Haut* gibt häufig unspezifische Resultate. Es ist bekannt, dass Allergien des Magen-Darmtraktes durch Hautreaktionen meistens nicht erfasst werden können. (4) Der *Haemagglutinations*-Test, den wir vor einigen Jahren für den Nachweis von Nahrungsmittel-Antikörpern eingeführt haben[1,2], hat trotz der von anderer Seite[3,4] erfolgten Prüfung und Bestätigung keine weitere Verbreitung gefunden. Mit anderen Worten: Von den *sub* 1–3 angeführten Methoden entspricht hinsichtlich des Sensibilitätsgrades sowie der Spezifität nicht eine den Ansprüchen kritisch eingestellter Kliniker, während man über den Wert des Haemagglutinationstestes wegen seiner bisherigen geringen Verwendung keine sichere Aussage abgeben kann.

Angesichts des in der praktischen Bedeutung der angeführten serologischen Verfahren begrenzten Wertes für den Nachweis von Nahrungsmittel-Antikörpern entschlossen wir uns eine 5. Methodik zu benutzen, welche eine Variante der ersterwähnten darstellt.

Für unsere Untersuchungen bedienten wir uns des Agardiffusionstests nach OUCHTERLONY[5], der in der Serologie der letzten Jahre in zunehmendem Masse Anwendung gefunden hat. Aus dem Gros der hier zu erwähnenden Versuche seien nur die von HAINER et al.[6] mittels des Ouchterlony-Testes vorgenommenen Studien über Antikörper gegen Kuhmilch, sowie gegen Gliadin erwähnt, in denen unsere früheren, mittels der Komplementbindungsreaktion sowie des Haemagglutinationstestes erhaltenen Befunde bestätigt wurden.

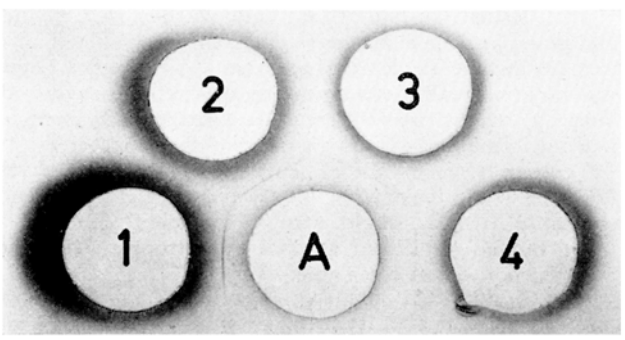
Methoden und Material. Wir benutzten eine Mikromodifikation auf Objektträgern des Agardiffusionstests nach OUCHTERLONY[5], bei der man mit 0,2 ccm Serum und 0,1 ccm Antigenlösung auskommt. In das auf die Objektträger ausgegossene 2% Agargel (2,5 ccm Agar pro Objektträger) wurden fünf runde Löcher mit einem Durchmesser von 9 mm gestanzt. Der Abstand der Löcher voneinander war 5 mm. In die vier um das zentrale Antigenreservoir gelegenen Löcher wurde das Serum in einer Verdünnungsreihe eingefüllt (Figur). (a) *Agar.* Der 4% in *aqua dest.* gelöste Difco-Agar wurde mit Veronalpuffer von pH 7,4 im Verhältnis 1 : 1 verdünnt. (b) *Serum.* Es wurde bei 56° 30 min inaktiviertes menschliches Serum von Neugeborenen (Nabelschnurblut), bei Kindern und Erwachsenen verwendet. (c) *Antigen.* Die 3,3% Lösung von Casein (nach Hammarsten) in verdünntem NaOH wurde mit n/10 HCl auf pH 7,2 gebracht. (d) *Färben.* Die getrockneten Präparate wurden mit Amidoschwarz gefärbt.

Ergebnisse. Die caseinpraezipitierende Antikörper enthaltenden Seren ergaben zwischen den mit Antigen und Serum gefüllten Löchern eine deutliche *Praezipitationslinie*, meistens bis zu einer Serumverdünnung von 1:8 (Figur). Bei der umgekehrten Versuchsanordnung (unverdünntes Serum in der Mitte, Antigen in einer Verdünnungsreihe in den äusseren Löchern) wurden mit den meisten Seren die gleichen Titer erzielt.

Zwecks Orientierung über den Wert des Ouchterlony-Testes für den Nachweis von Casein-Antikörpern untersuchten wir 235 Seren von 170 Personen sowohl mit der Haemagglutinationsreaktion als auch mit dem Ouchterlony-Test auf Casein-Antikörper. Die mit den beiden Methoden erhaltenen Befunde sind in Tabelle I wieder-

Tab. I. Der Nachweis von Casein-Antikörpern. Vergleich der mit dem Hämagglutinations- und dem Ouchterlony-Test erhaltenen Resultate in 235 untersuchten Seren

Ouchterlony	positiv		negativ		total
	19 Seren		216 Seren		235 Seren
Haemagglutination	positiv	negativ	positiv	negativ	total
	12	7	68	148	235 Seren



Der Nachweis von Casein-Antikörpern im Agar. A 3,3% Caseinlösung, 1 unverdünntes Serum, 2 1:2 verdünntes Serum, 3 1:4 verdünntes Serum, 4 1:8 verdünntes Serum.

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