Histopathological modifications following immunotherapy with P 40 in rats bearing DMBA-induced mammary carcinoma[1,2]

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Summary. Female Sprague-Dawley rats bearing primarily-induced mammary carcinomas were treated with a new immunostimulating agent (P 40). The histopathological modifications encountered in tumors, lymph nodes, liver and spleen are described. Intratumoral injections provoke a more widespread response of the RES, and particularly a more intense reaction in the draining lymph nodes, than systemic treatment.

Numerous investigators have shown that various immunostimulating agents inhibit tumor growth in experimental systems[3-12]and in some human tumors[13]. Several authors have reported that intratumoral administration gives better results than systemic treatment by i.v. injection[8-10,14,15]. However, the histopathological basis of the observed differences is not clearly established.

The aim of the present report is to deal with histopathological modifications observed in animals bearing primarily-induced (and not grafted) mammary carcinoma, treated by immunotherapy when the tumors had attained a diameter of 0.5-2.5 cm. Thus, in the present model the natural lymphatic drainage from the tumor to the regional lymph nodes is preserved and the immunostimulator is administered at a late stage, and not, as in other studies before, together with or shortly after cell grafting.

The immunostimulator used in this study is a new agent from the Pasteur Institute, Paris, which was isolated from *Corynebacterium granulosum,* strain 5196[16]. This compound called P 40 was obtained by fractionation of delipidated whole cells and is characterized by its great resistance to all kinds of enzymatic and chemical degradation. It is not altered by heating at 120 °C for 20 min and can thus be sterilized without addition of formalin.

The animal model used was the DMBA-induced rat mammary tumor, as described by Huggins et al.[17]. This system provides primarily induced tumors known for their individual antigenicity. Sprague-Dawley female rats, kept under standardized laboratory conditions, received at the age of 55 days, by gastric instillation, a single dose of 18- 20 mg of 8,10-dimethyl-1,2-benzanthracene (DMBA) dissolved in sesame oil. The mortality was about 30% and 90-95% of the surviving animals developed mammary tumors between the 7th and the 17th week after administration of the carcinogen. Thus, 55 tumor-bearing animals were obtained for the 1st study. 45 animals were treated with P 40, either by local (intra- or peri-tumoral) or by i.v. injections, and 10 animals served as controls without treatment or after injection of saline. P 40 was administered on 3 following days and the doses per injection varied from 100 to 4000 µg per animal. If several tumors were present the doses were equally distributed between them. Thus, individual tumors received 17-2000 µg of P 40 according to the number of tumors per animal (see table). 9 rats were sacrificed 9 days after the last injection and 36 animals 15 days after the last treatment. The controls were killed at the same time. Tissue samples from tumors, draining and controlateral lymph nodes, liver and spleen were fixed for histological and fine structural examination.

All mammary tumors induced by DMBA instillation were typical adenocarcinomas. The draining lymph nodes had a well preserved structure and showed a pronounced sinus histiocytosis. No lymph node or visceral metastases were observed.

After treatment with P 40 the histological modifications were always of the same type but their extension and their intensity varied depending on the route of administration. Thus, unexpectedly, the modifications were generally more intense and more widespread after local than after systemic treatment.

After local administration, i.e. intra- or peri-tumoral injection of P 40, the injected tumors showed necrotic alterations accompagnied by a diffuse histiocytic reaction. The non-injected tumors of the same animals were not modified. The draining lymph nodes of injected tumors were, in almost all cases, the site of a histiocytic reaction, which consisted of granulomas appearing in the cortical and paracortical regions (figure 1). These focal granulomas were composed of histiocytic-macrophagic elements (figure 2). In the cases of very strong reaction, nodular granulomas coalesced and the histiocytic reaction became diffuse and then entirely occupied the node. Furthermore the controlateral lymph nodes were sometimes the site of a moderate granulomatous response.

The spleens were markedly altered and showed numerous granulomatous foci in areas normally occupied by active germinal centers (figures 3 and 4). These changes were accompagnied by a pronounced erythroblastic and megakaryocytic reation. The granulomas were of the same type as in the lymph nodes. The liver was also involved in the histiocytic reaction. The focal granulomas were preferentially situated in the peri-portal fields (figure 5), but also occurred along the sinusoids. As shown previously by one of us[4], this kind of lesion induced by bacterial extracts apparently develops from the Kupffer cells.

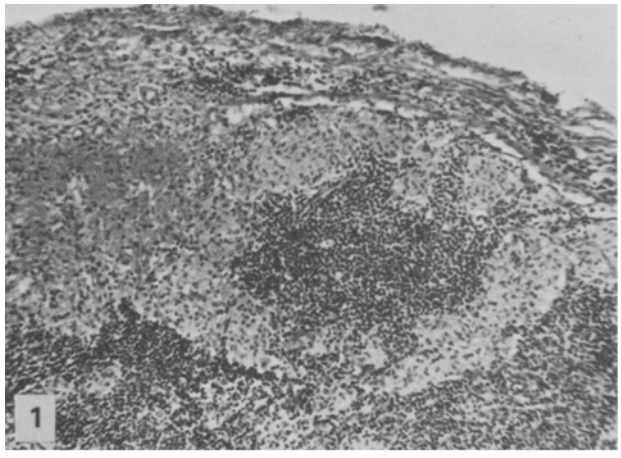
After i.v. injection of P 40, the liver and the spleen showed the same type and almost the same intensity of modifications as observed after local treatment, However, no response occurred in the tumors and in the draining lymph nodes.

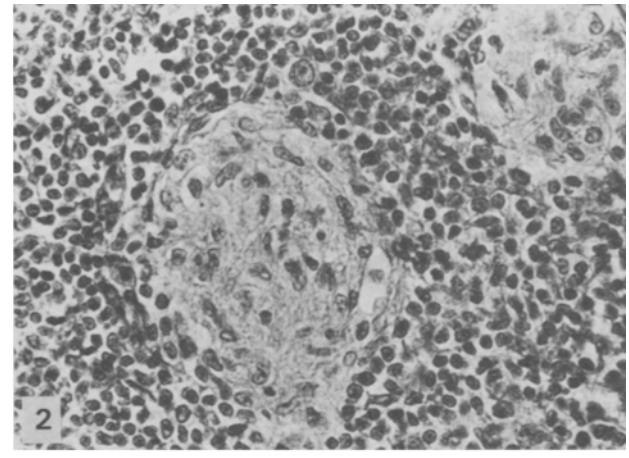
The intensity of the histiocytic reaction was closely related to the dose of P 40 injected. Doses below 100 µg of P 40 were almost ineffective. Doses of 500 µg sometimes produced large tumor necroses and considerable cellular reactions. But the most regular and intense responses were obtained with doses of 2000 µg per tumor. Intra- or peritumoral administration of the immunostimulating agent gave identical results for both the type and the intensity of the reaction.

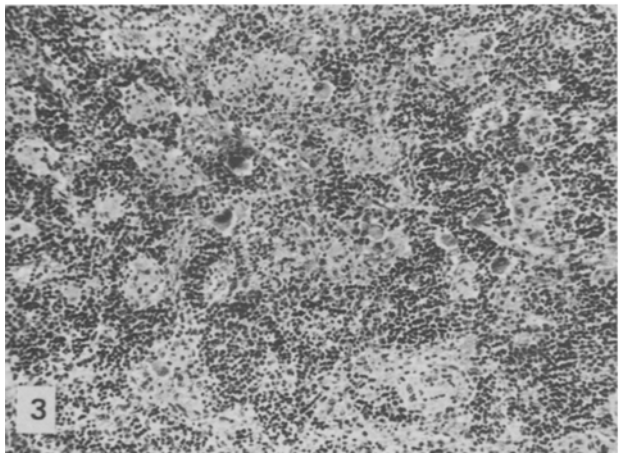
The present histopathological study confirms that P 40 had a strong immunostimulating effect on the RES as previously shown by Lallouette et al.[18] by the increase of anti-

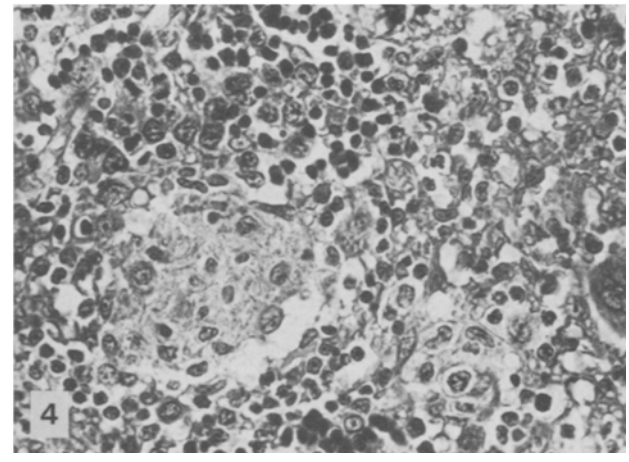
Immunotherapeutic regimen administered for 3 consecutive days

	Number of rats treated	Dosis per tumor and per day (µg)	Dosis per animal and per day (µg)
P40 intratumoral	6	17-100	100
	6	71-500	500
	6	333-2000	2000
	12	1500-2000	1500-4000
P40 peritumoral	7	1500-2000	1500-4000
P40 i.v.	8	-	2000









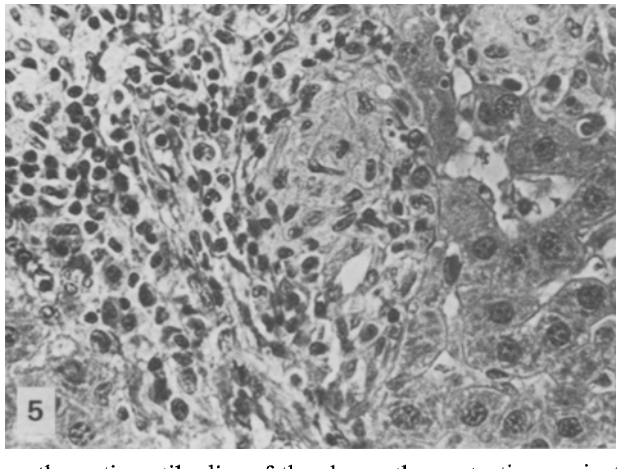


Fig. 1. Draining lymph node of locally P-40-treated mammary carcinoma. Coalescent granulomas in the cortical region. × 97.

Fig. 2. Draining lymph node of locally P-40-treated mammary carcinoma. 2 focal granulomas in the paracortical region consisting of large, pale-staining histiocytes. × 387.

Fig. 3. Spleen of P-40-treated mammary carcinoma bearing rat. Numerous histiocytic granulomas are scattered throughout the spleen. × 97.

Fig. 4. Spleen of P-40-treated mammary carcinoma bearing rat. At higher magnification the granulomas have the same structure as those encountered in the lymph nodes. × 387.

Fig. 5. Liver of P-40-treated mammary carcinoma bearing rat. 2 histiocytic granulomas in the vicinity of a peri-portal field. × 387.

erythrocytic antibodies of the sheep, the protection against infectious agents and the inhibition of tumor growth. As in the case of other immunostimulating agents of bacterial origin, the action is correlated with an activation of macrophagic elements as demonstrated by measuring the blood clearance of colloidal carbon after injection of P 40[18].

Histopathologically, the stimulation of the RES consists in a reaction of histiocytemacrophages of a granulomatous type as described by other authors using other immunopotentiating agents and studying either the local reaction after intra- or peri-tumoral injection[5,7] or the systemic reaction after i.v. or i.p. administration[4]. A major point of interest in our study, however, is the demonstration of the definite difference of the action of the immunostimulator according to the method of administration. Thus, systemic injection of P 40 induces a marked proliferation of histiocytes in the liver and the spleen without modifications in the lymph nodes and the tumors, whereas local injection, either intra- or peri-tumorally, induces a widespread granulomatous response affecting not only the liver and the spleen, but also the injected tumors and the regional lymph nodes.

The role of the route of injection of the immunostimulator on tumor growth has already been recognized by several authors. Most of them reported that intratumoral administration more effectively inhibits tumor growth than systemic treatment does[5–7,11–13]. Greager and Baldwin[3] treated

transplanted mammary carcinoma bearing rats with intratumoral or i.v. injections utilizing either BGG or *Corynebacterium parvum*. They obtained the most significant results in animals treated with repeated intratumoral injections of *C. parvum*.

Likewise, Fisher et al.[14], found that the intratumoral inoculation of *C. parvum* more effectively inhibited grafted tumors than did its use by any other route.

Although the advantage of the intratumoral route versus other routes had already been observed, the reasons for this discrepancy were not yet clearly understood. The present histological findings clearly demonstrate that intratumoral injection provokes a much more widespread response of the RES than systemic treatment and thus provides a morphological basis for further studies on the action of immunostimulating agents.

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Visualisation of lectin binding sites on the surface of human platelets using lectins adsorbed to gold granules[1]

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Summary. Washed human platelets have been incubated with the lectins WGA, ConA and RCA_1, adsorbed to different-sized gold particles. Plasma membrane receptors for each lectin were then located by scanning and transmission electron microscopy.

The presence of platelet functional defects in patients with congenital platelet disorders associated with specific abnormalities of different membrane glycoproteins[2,3] strongly suggests a role for the membrane glycoproteins in the physiologic mechanisms of platelet aggregation and adhesion. Lectins are proteins with different specificities for the sugar residues commonly found in glycoproteins and glycolipids. Certain lectins are able to activate platelets, stimulating both the release of the contents of intracellular storage organelles[4,5] and platelet aggregation[4,6]. Among such lectins are wheat germ agglutinin (WGA), concanavalin A (ConA) and *Ricinus communis* lectin (RCA_1). Horisberger and Rosset[7] have developed a procedure for the localization of cell surface glycoconjugates by scanning and transmission electron microscopy using lectins adsorbed to gold granules. In the present study we have used this procedure to investigate the distribution of WGA, ConA and RCA_1 receptors on the human platelet surface. This approach was thought potentially useful as the small size of the blood platelet (2–4 µm in diameter) makes conventional fluorescence microscope procedures using fluorescein-labelled lectins difficult to perform, and relatively little is known about the organisation of the individual components of the platelet glycocalyx.

Material and methods. 1. Preparation of fixed, washed platelet suspensions. Blood (9 vol.) was taken from adult human donors directly into the acid-citrate-dextrose anticoagulant of Caen et al.[8]. Prostaglandin E_1 (PGE_1) (100 nM) was added to each of the solutions used during the washed platelet isolation, which was performed using a modification of the procedure of Levy-Toledano et al.[9]. A stock solution of Metrizamide (Nyegaard, Oslo, Norway) in water at 300 mosm was diluted with 0.02 M sodium phosphate buffer pH 5.7, containing 0.139 M NaCl and 0.01 M glucose to give solutions of 25% and 10% w/v Metrizamide respectively. Step gradients were prepared by overlayering 1 ml 25% Metrizamide with 2 ml 10% Metrizamide in 10-ml plastic tubes. Each interface was slightly stirred using a looped steel wire. An aliquot of platelet-rich plasma ($1.5–2.0 \times 10^9$ platelets) was then added to each tube. Centrifugation was performed at $2000 \times g$ for 15 min at room temperature. The platelets, which sedimented to the 10%/25% Metrizamide interface, were resuspended in 0.02 M sodium phosphate buffer, pH 7.4, containing 0.139 M NaCl and 0.01 M glucose at $0.5 - 1.0 \times 10^9$ platelets ml^{-1}. Glutaraldehyde (4% w/v in pH 7.4 buffer) was added in an equal volume and the platelet suspension incubated at room temperature for 2 h. The fixed platelets were sedimented, resuspended in 0.01 M tris buffer, pH 7.4, containing 0.15 M NaCl, and washed twice.

2. Marking of platelets with lectin-labelled gold granules. Gold (Au) granules of different sizes (5, 12, 32 and 50 nm) were prepared as described by Horisberger and coworkers[7,10]. The code following each Au-marker in the text refers to the average diameter of the particles. The lectins used were WGA (Pharmacia Fine Chemicals), ConA (Miles laboratories) and RCA_1 (mol.wt 120,000) (Sigma, type II). WGA and RCA_1 were cross-linked to bovine serum albumin prior to the labelling of the granules[7]. The gold particles were labelled by incubation at 25 °C in the