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MORPHOLOGY OF DRAINING LYMPH NODES AFTER LOCAL IMMUNE STIMULATION WITH *C. PARVUM*: COMPARISON OF PELVIC NODES IN CARCINOMA OF CERVIX AND POPLITEAL AND INGUINAL NODES OF GUINEA-PIG

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Summary.—Morphological changes are described in pelvic lymph nodes excised 10 days after *C. parvum* (CP) treatment of patients with cervical carcinoma. Guinea-pig popliteal and inguinal lymph nodes were investigated from Days 1 to 10 after an injection of 70 μg CP into the footpad. Eosinophils were detected from the first few hours after stimulation, initially in the marginal sinus, then in the medullary sinuses and subsequently in the efferent lymphatics. From Day 2 to Day 6, histiocyte accumulations with the appearance of epithelioid cells were found mainly in subcapsular and interfollicular areas, and small granulomas were also seen in the paracortex.

The granuloma formation in the lymph node was considered as an indication of the activation of histiocytes. Besides small granulomas in the paracortex, activated interdigitating cells, surrounded by scattered lymphoblasts and eosinophils, were also present. We considered this lymphoblastic response and eosinophilic accumulation as likely to be due to blastogenic factor and eosinophil stimulation promotor. Eight to 10 days after CP stimulation, the macrophage lymphoblast eosinophil response was replaced by a B-cell reaction: germinal-centre activation and medullary plasma cells. Such a B-cell reaction was also found in the human pelvic nodes removed at operation, but this reaction could not be attributed to CP treatment alone, since cervical-carcinoma patients not treated with CP also showed such reactions. In contrast, pelvic lymph nodes removed at necropsy from females killed in traffic accidents showed no predominance of either B- or T-cell stimulation.

A RELATIVELY SMALL DOSE (2 mg) of the immune stimulant *Corynebacterium parvum* was given as a single injection in the neighbourhood of a cervix carcinoma 10 days before excision of the tumour by radical surgery. Such treatment induces longer postoperative relapse-free intervals, as well as lower relapse rates. The immunological functions of peripheral lymphoid cells tested in these patients suggest that the clinical benefit is brought about by an improved macrophage and T-cell function (Mignot *et al.*, 1981). BCG, one of the most extensively studied immune stimulants, induces clear macrophage reactions in draining lymph nodes as sinus histiocytosis and granuloma formation (Gaafar & Turk, 1970; Hanna *et al.*, 1972; Khalil *et al.*, 1975). The T- and B-cell compartments are stimulated as well: lymphoblasts appear in the paracortex as a sign of T-cell stimulation, whereas later germinal centres are seen in the cortex, and plasma cells in the medulla, as signs

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of B-cell stimulation (Gaafar & Turk, 1970; Khalil *et al.*, 1975).

The combination of radical surgery and preoperative local immune stimulation provided, in addition to an improvement in the management of cervical-cancer patients, an opportunity to investigate the draining lymph nodes.

A comprehensive study of the time course of lymph-node reactions after local CP stimulation is absent from the literature. For this reason, a time-course study on local CP stimulation in an animal model was performed. Guinea-pig popliteal and inguinal lymph nodes were studied from 6 h to 10 days after injecting the footpads with 70 μg of CP. The histological responses of these lymph nodes were compared with those of the pelvic lymph nodes removed at operation from cervical cancer patients and those of healthy controls.

MATERIALS AND METHODS

Guinea-pigs.—Female guinea-pigs of the Hartley strain (TNO Zeist, the Netherlands) weighing 350–450 g were used. The animals were fed on a diet of pellets supplemented with cabbage.

Patients.—The pelvic lymph nodes of 16 patients suffering from squamous-cell carcinoma of the uterine cervix, Stage I^B (Kottmeier *et al.*, 1979) were investigated. Median age was 49 years (range 35–62). The patients were randomized for age and allocated into two treatment groups using a computer-generated pseudo-random table. One group consisting of 8 patients was treated with the immune stimulant CP, whilst the other received no immune therapy.

No metastases were evident at operation in any of these patients.

C. parvum *administration.*—The CN6134 strain of *Corynebacterium parvum* (Wellcome Laboratories, Kent; CP) was used in all cases. In guinea-pigs, half the animals received 70 μg CP in 0·1 ml physiological saline containing 0·01% thiomersal into the right hind footpad. The 70 μg is the equivalent of the 2mg dose of CP used in our clinical studies (Wellcome, 1978). The other half of the guinea-pigs were used as controls and received 0·1 ml of physiological saline (0·01% thiomersal) only.

The patients received 2 mg of CP in 0·2 ml physiological saline (0·01% thiomersal). The dose was divided into 4 aliquots, given as 4 neighbourhood injections around the tumour, 10 days before radical surgery.

Lymph nodes.—The popliteal and inguinal nodes of the experimental animals were removed 6 h and 1, 2, 4, 6, 8 and 10 days after the footpad injection. Comparison was made between the lymph nodes removed from the CP-injected animals and the saline-injected controls. In the patients with carcinoma of the cervix, selected lymph nodes which had been removed from the pelvic region, including the parametric region, the region of the arteria iliaca externa, the iliaca interna, the iliaca communis and the fossa obturatoria were examined. Generally one lymph node from each subregion was investigated.

Comparisons were made between the lymph nodes of CP-treated patients and those not receiving immune stimulation.

In these investigations we included the pelvic lymph nodes of 5 healthy women killed in traffic accidents. Their mean age was 32 years (range 26–42).

The lymph nodes of both experimental animals and patients were fixed in a mixture of formaldehyde, acetic acid and mercuric chloride for 2–3 h at 4°C, as described by Romeis (1968). They were then treated with Lugol solution and 5% sodium thiosulphate, dehydrated, embedded in paraffin wax, sectioned at 5 μm and stained with Giemsa stain, haematoxylin–eosin and methyl-green–pyronin.

Analysis of lymph-node histology.—In guinea-pigs, the enlargement of the lymph node, the paracortex, the follicles and germinal centres were measured by histomorphometrical techniques using a Leitz ASM analysis system as described by Boon *et al.* (submitted). Each lymph node was semi-serially sectioned at 5 μm; 3 sections, at $\frac{1}{4}$, $\frac{1}{2}$ and $\frac{3}{4}$ of the depth of the node, were taken for histomorphometrical analysis. The numbers of lymphoblasts in the paracortex and of plasma cells in the medulla were determined by counting them in a randomized fashion in 10 oil-immersion fields ($\times 100$) of sections stained with methyl-green–pyronin. The distribution and the number of eosinophils in the guinea-pig lymph nodes

were evaluated semiquantitatively. Sections of the human pelvic lymph nodes were evaluated similarly.

Statistical analysis.—The statistical analysis for comparison of the control and the immune-stimulated groups of lymph nodes used Fisher's 2×2 exact test or the Willcoxon 2-sample test.

RESULTS

Guinea-pig lymph nodes within 2 days of CP treatment

A few hours after the injection of 70 μg CP into the footpad, eosinophilic leucocytes were seen in the marginal sinus of the popliteal node, and by 6 h they constituted 10–15% of the cellular population in the sinus. In the saline-injected controls no such reaction was seen. Within 24–48 h the eosinophils were seen in the medullary sinuses, and thereafter they were visible in the efferent lymphatics.

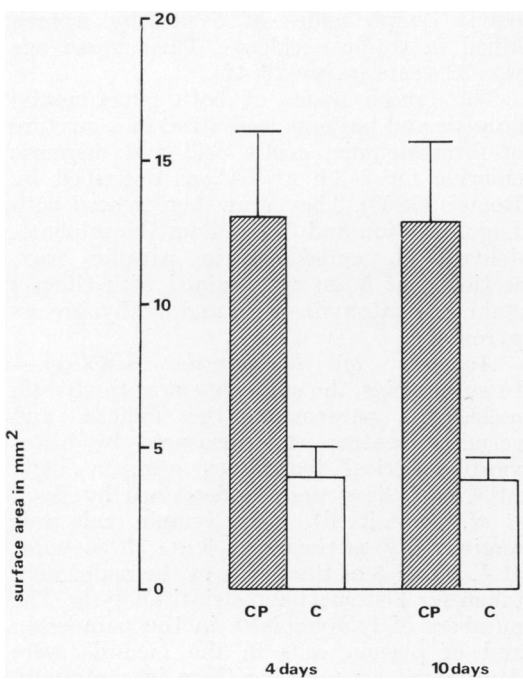

FIG. 1.—Enlargement of the popliteal lymph node, 4 and 10 days after 70 μg *C. parvum* administered to the hind footpad of guinea-pig in CP-treated animals ($n = 5$) and controls (C, $n = 5$).

The inguinal lymph nodes showed a similar influx of eosinophils, but the reaction was observable only after 24 h and was much milder.

Guinea-pig lymph nodes 2–6 days after CP treatment

Fig. 1 shows the analysis of the enlargement of the popliteal lymph node 4 and 10 days after CP stimulation. The areas of the popliteal-node sections increased to over 3 times the areas of the nodes in the saline-injected controls. This increase in size of the nodes was initially seen 2–3 days after immune stimulation, and reached its maximum on Day 4. The nodes stayed enlarged throughout the 10 days of observation. A similar enlargement was found in the inguinal nodes, though the size was only twice that of the controls.

The most remarkable feature after CP stimulation was the activation of the mononuclear phagocyte system in the lymph node. At 2–6 days, histiocytes were present mainly in a subcapsular and interfollicular position in the cortex of the popliteal node, especially in the draining area of the afferent lymphatics (Fig. 2). Granuloma formation with the appearance of epithelioid cells occurred, but no giant-cell formation could be detected. At the centre of these granulomas there were usually areas of necrosis containing polymorphonuclear leucocytes (Fig. 3).

Occasionally we saw phagocytosis of these cells. Small granulomas of 2–4 cells were found in the paracortical area on Day 4 (Fig. 4a). These granulomas were associated with large macrophage-like cells with elongated protrusions. These macrophage-like cells were localized at the position of the antigen-handling interdigitating paracortical cells described by others (Fig. 4b) (Hoefsmit, 1975; Kamperdijk, 1980). These small granulomas and the interdigitating cells were surrounded by scattered large pyroninophilic lymphoblasts: some of them in mitosis. The quantitative analysis of lymphoblasts in the paracortical area is given in Fig. 5. A significant increase in their number was

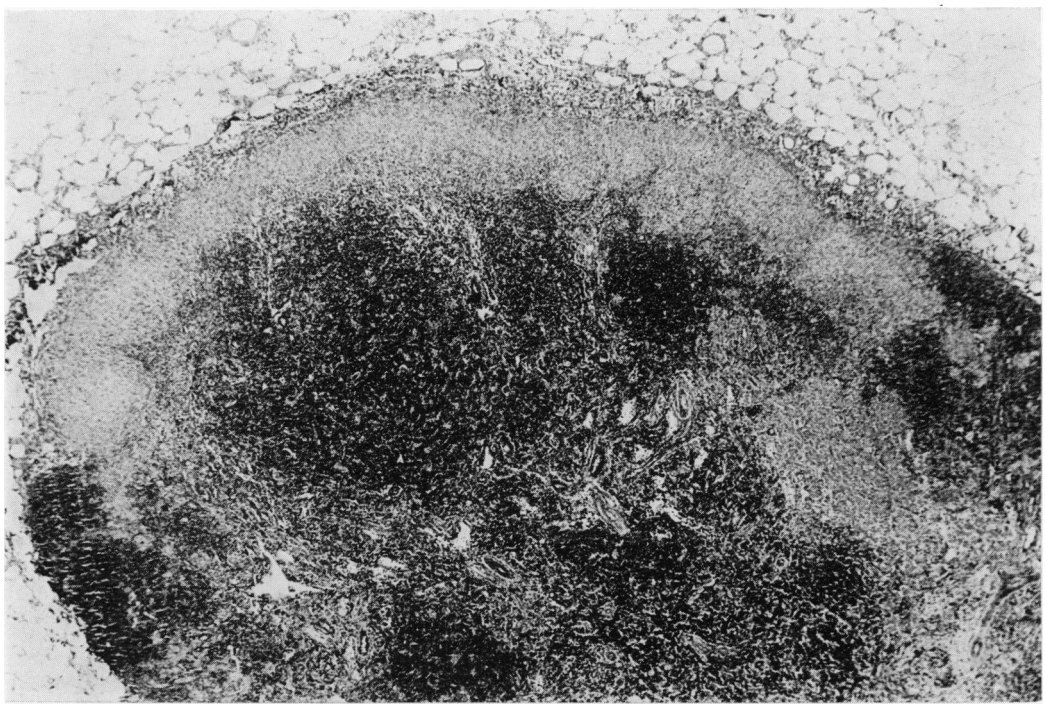

FIG. 2.—Morphology of the popliteal lymph node of the guinea-pig 4 days after the administration of 70 μg CP to the hind footpad. Sinus histiocytotic and granulomatous reactions, especially in the subcapsular and interfollicular region of the afferent lymphatic, are apparent. Giemsa × 2·5.

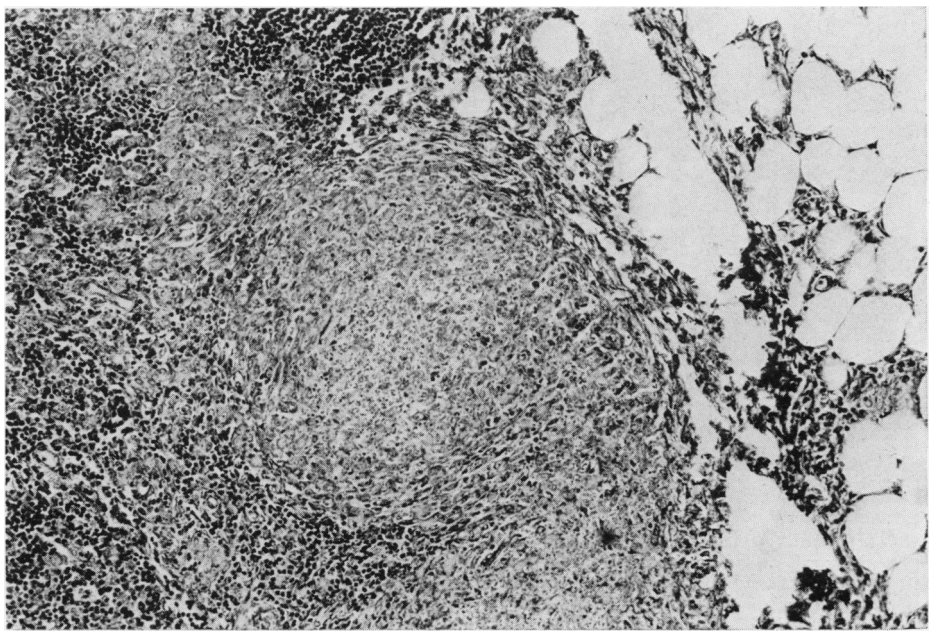

FIG. 3.—Small foci of necrosis in the granulomatous areas in guinea-pig lymph nodes, 4 days after local CP administration. Cytophagocytosis inside these necrotic areas is apparent. Giemsa × 10.

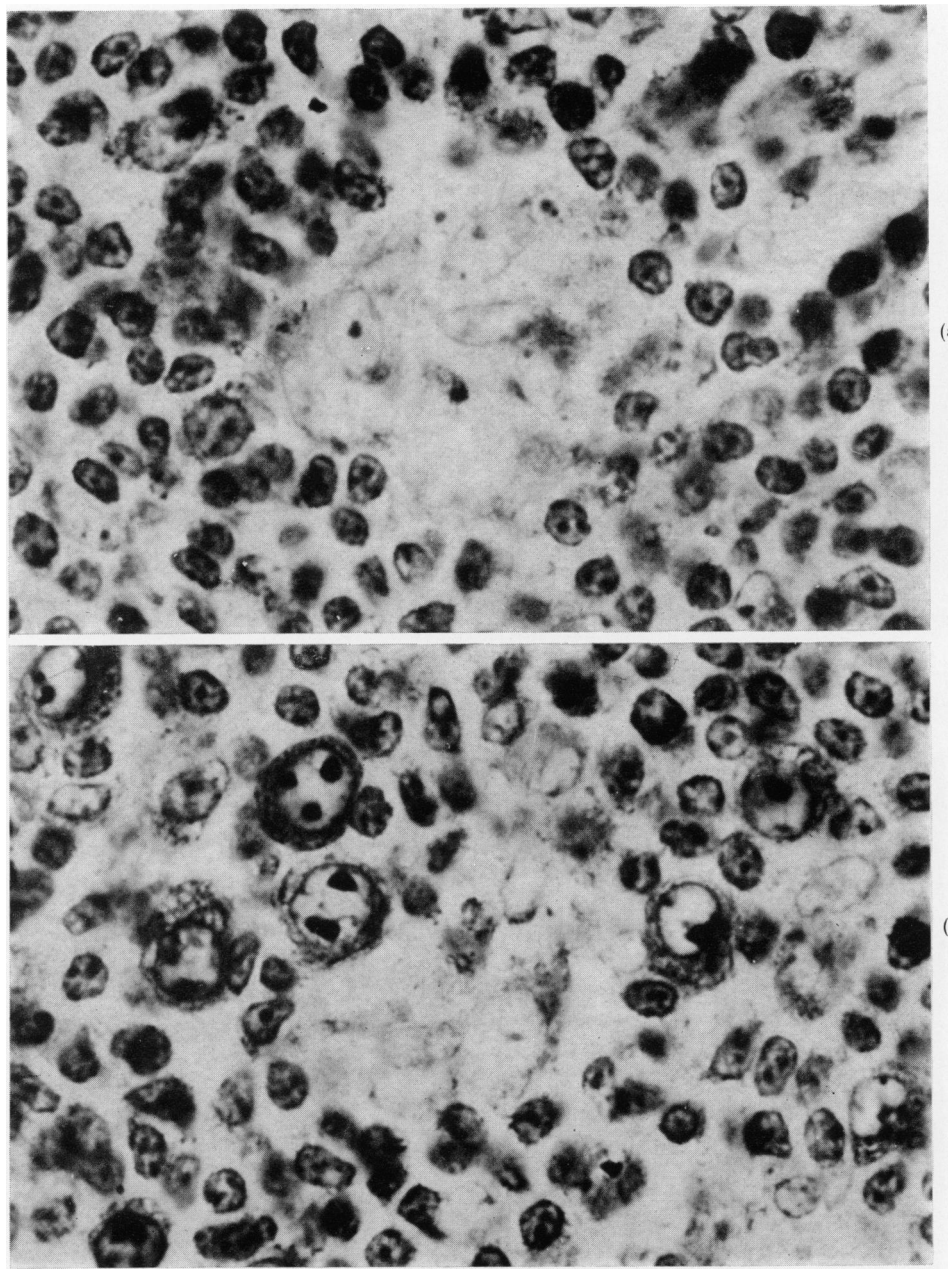

FIG. 4.—(a) Small granuloma of ~5 macrophage-like cells in the paracortical area of a guinea-pig popliteal lymph node 6 days after the administration of 70 μg of CP to the hind footpad. Giemsa $\times$ 100. (b) Pale, swollen macrophage-like cells at the position of the interdigitating cells in the paracortical area of guinea-pig popliteal node 6 days after the administration of 70 μg of CP to the hind footpad. The cells are surrounded by scattered numerous lymphoblasts.

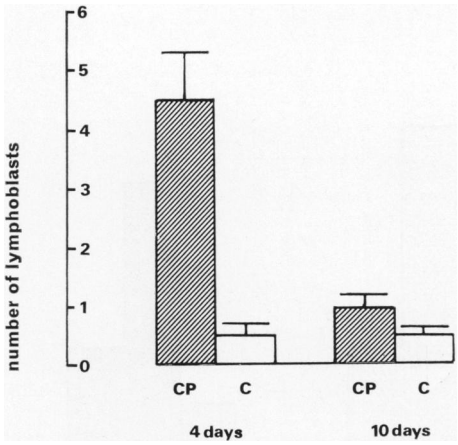

FIG. 5.—Numbers of lymphoblasts in the paracortical area of the popliteal lymph node 4 and 10 days after the administration of 70 μg of CP to the hind footpad of the guinea-pig.

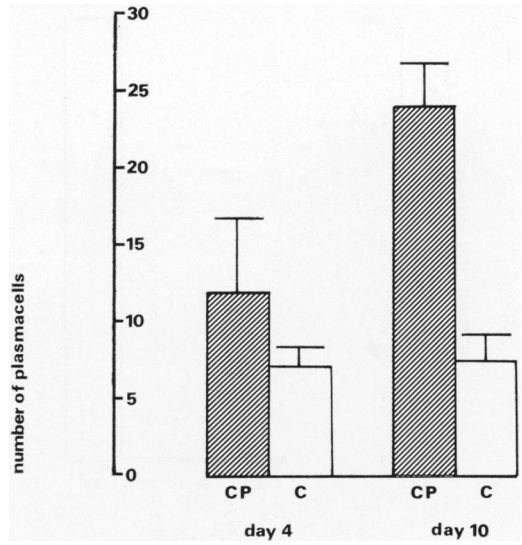

FIG. 6.—Numbers of plasma cells in a $\times$ 100 microscopic field of the medulla of a popliteal lymph node 4 and 10 days after the administration of 70 μg of CP to the hind footpad of the guinea-pig.

readily noticed 4 days after CP stimulation. Eosinophils were scattered around these areas of macrophage and lymphoblastic activity, especially in the subcapsular cortical area and in the immediate vicinity of the lymph-node outer capsule. The maximal eosinophilic response coincided with that of the macrophage/lymphoblastic response, namely 4–6 days after CP administration. The inguinal lymph nodes showed a similar type of macrophage / lymphoblastic / eosinophilic response, but the maximal reaction was one-third of that in the popliteal lymph nodes.

Guinea-pig lymph nodes 8–10 *days after CP treatment*

Granuloma formation and paracortical lymphoblastic response had by now disappeared completely. Of the macrophage reaction, very few scattered patches of histiocytes were seen. The general enlargement of the lymph node (see Fig. 1) was due to enlargement of the follicles, with a concomitant extensive germinal-centre reaction. A large number of plasma cells were found in the medullary cords (Fig. 6). By Day 10 the plasma-cell reaction reached its maximum, at twice the control value. The inguinal lymph nodes showed a similar reaction, but reached only three-quarters that in the popliteal lymph node.

Human pelvic lymph nodes 10 *days after CP treatment*

The morphological pattern of the human pelvic lymph nodes after stimulation with 2 mg CP appeared less homogeneous than that in guinea-pig lymph nodes. In most of them small to extensive areas of infiltrated fat cells, hyalinization and fibrosis were seen. The histomorphometrical measurement of the paracortex, the follicles and other compartments was therefore extremely difficult. However, there was clearly a moderate sinus histiocytosis in almost all the nodes of the patients, with no detectable difference between the untreated and treated patients. Additional semiquantitative measurements could detect no difference in number of paracortical lymphoblasts, nodular plasma cells or extension of the germinal-centre reaction between these groups. As in another study on pelvic lymph nodes of cervical-cancer patients (Tsakraklides *et*

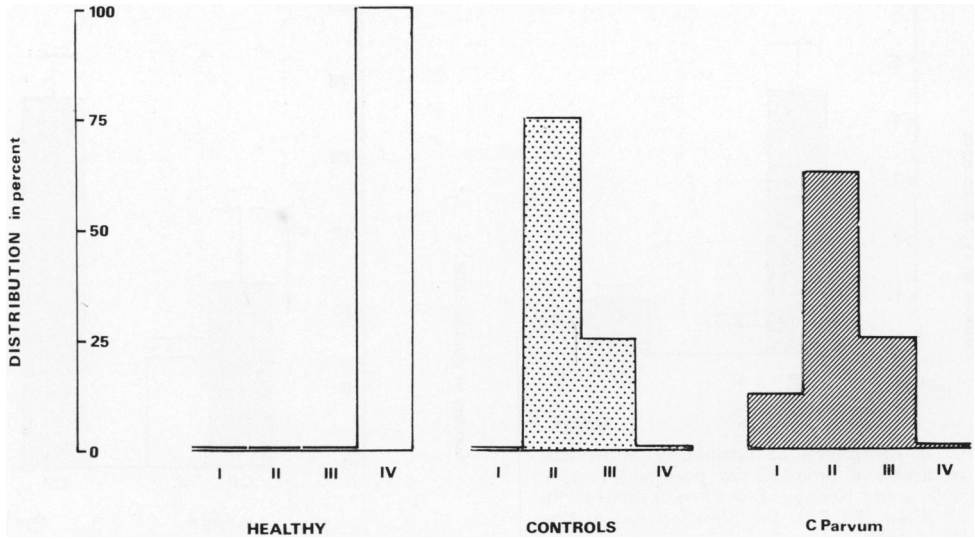

FIG. 7.—Population histograms for the 4 morphological patterns found in draining lymph nodes of the cervix uteri. Comparisons are shown between the pattern in healthy females killed in traffic accidents ($n=5$), carcinoma-cervix patients ($n=8$) and carcinoma-cervix patients treated with local *C. parvum* ($n=8$). I = Mainly paracortical stimulation. II = Mainly germinal-centre formation. III = Mainly degenerative characteristics, such as hyalinization and fibrosis. IV = Unstimulated nodes without signs of degeneration. No statistical difference was found between the untreated and CP-treated patients (2×2 exact test).

al., 1973), 4 types of different morphological patterns could easily be distinguished, namely: (a) nodes with predominant paracortical-area stimulation, (b) nodes with clear germinal-centre reactions as plasma-cell reactivity, (c) nodes with extensive degenerative characteristics such as hyalinization and fibrosis and (d) nodes with practically no areas of degeneration, but showing no immune stimulation. On each patient at least 5 nodes, each from a different subregion, were studied, and thereafter the patient was classified according to the type of node found in at least 60% of them. Results are shown in Fig. 7. Again, no statistically significant differences could be detected between the two groups of patients, though there was 1 patient in the CP-stimulated group who showed a paracortical predominance. In the other group no such stimulation was found. The most interesting finding in this human study, however, was the difference between the nodes of the carcinoma-cervix patients and of a group of 5 healthy women, none of whose nodes showed any degenerative characteristics or immune stimulation (Fig. 7), whereas most of the nodes of the carcinoma patients had a strong plasma-cell reaction and concomitant germinal-centre reactivity.

DISCUSSION

The most marked nodal reaction after a single footpad injection of 70 μg CP into guinea-pigs is a transient reaction of the mononuclear phagocyte system in the popliteal and inguinal lymph nodes. Epithelioid cells appear and granulomas with foci of necrosis are formed, primarily in the subcapsular and interfollicular sinuses, and also in the paracortical areas. Giant cells could not be found. Studies on the nitroblue tetrazolium-dye reduction capacity of histiocytes isolated from these nodes showed a higher reducing capacity than histiocytes from normal lymph nodes, indicating enhancement of their hexose-monophosphate shunt (Mignot, 1982). Reducing equivalents generated in this

shunt are used to produce the hydrogen peroxide necessary for the destructive capacity of the macrophage (Lace *et al.*, 1975; Johnston *et al.*, 1980). The interdigitating cells, the antigen-handling macrophages of the paracortex, are also stimulated by local CP injection. Interdigitating cells are known to enter the lymph node *via* the afferent lymphatic as veiled cells (Drexhage *et al.*, 1979), while originating from the site of injection as the antigen-bearing Langerhans cells (Silberberg-Sinakin *et al.*, 1974). In our study, the interdigitating cells, with their lucent cytoplasm and extended protrusions, were clearly visible and surrounded by scattered pyroninophilic lymphoblasts, some of them in mitosis. The morphological picture represents the stimulation of the T-cell system, probably *via* a blastogenic factor generated by the interaction of T cells with antigen on the surface of the interdigitating cell. Immune-peroxidase-labelling experiments have shown the presence of CP antigens on the surface of these cells, observations which will form the subject of our next report.

Lymphokines, other than blastogenic factor, are probably also generated in the interaction; the accumulation of eosinophils 6–8 days after CP injection in the vicinity of the paracortex is most likely due to the generation of eosinophil stimulation promotor (ESP) (Weller & Goetzl, 1979). The eosinophilic accumulation in the first few days after CP injection is more puzzling. Other studies from our laboratory on the cell traffic in lymphatics draining a tumour area in the rabbit ear showed also up to 90% of eosinophils the first 24 h after CP injection (Schuitemaker *et al.*, to be published). It has been reported that pneumococcal antigens can evoke recompartmentalization of eosinophils through what is known as eosinopenic factor (EP) (Bass, 1975, 1977). CP might well exert similar effects. It is not known whether this early accumulation of eosinophils in the node has any effect on the later events.

BCG has immune-stimulating effects similar to those of CP. It also induces macrophage reactions in the draining node, though these reactions have later onsets, namely at 6–14 days (Gaafar & Turk, 1970; Hanna *et al.*, 1972; Khalil *et al.*, 1975) and they are much more prolonged (up to 85 days; Khalil *et al.*, 1975). The collection of histiocytes in these BCG reactions may increase considerably in size, and sometimes the whole node is almost completely replaced by histiocytes (Gaafar & Turk, 1970). This is probably because BCG is live vaccine, whereas CP is dead.

Lymph-node histiocytes activated by nonspecific immune stimulants can be cytopathic to tumour cells. Cinematographic studies pictured these histiocytes moving aggressively about the surfaces of tumour cells without phagocytosing them (Snoddgrass & Hanna, 1973). The cytopathic mechanism by which such "activated" macrophages destroy tumour cells is not completely understood, though sizeable areas of apparent fusion are found, lending support to the idea that the cytopathic effect is mainly a contact phenomenon (Woodruff, 1980). Besides a cytopathic effect, activated macrophages and especially activated interdigitating cells, stimulate the antigen-specific T-cell response. In support of this view, we found an enhanced DNCB skin test, a raised number of E rosettes and a greater blastogenic transformation to PHA (Mignot, 1982) in patients treated with local CP stimulation. In our studies on the pelvic lymph nodes of patients with carcinoma of the cervix, we were unable to detect a morphological reaction to CP stimulation, though patients had raised E-rosette counts and enhanced DNCB skin tests. Boak (1978) did report histological changes after an injection of 0·5–7·0 mg of CP in carcinoma of the breast: the ipsilateral axillary lymph nodes were enlarged when removed after 9–18 days, and demonstrated marked sinus histiocytosis, though the histiocytes were unable to prevent the development of lymph-node metastases. Such an effect

had been described for BCG (Hanna *et al.*, 1972; Snoddgrass, 1973; Bast *et al.*, 1974).

Although we are well aware of fundamental differences between our animal and human studies ,we are convinced that we would have encountered in the human nodes at Day 10 a marked germinal-centre and plasma-cell reaction. The maximum histiocytic response should by then have subsided. We did find a moderate sinus histiocytosis and marked germinal-centre and plasma-cell reactions, which were no stronger than in the lymph nodes draining these types of carcinoma, as became evident from the results in our untreated patients. Additionally, it is possible that the morphological reaction to CP was masked by a strong reaction to antigens of the commensal vaginal flora. In support of this view the carcinoma-cervix patients showed markedly enhanced *Candida* and streptokinase/streptodornase skin-test responses (Mignot, 1982), both antigens from organisms commonly present in the vagina (Henley *et al.*, 1974; Brown, 1978; Tashjian, *et al.*, 1976) and which may easily gain entrance *via* the malignant ulcerations. It is also possible that the removed pelvic lymph nodes were not the first draining stations (Reifenstuhl, 1957, 1967). Unfortunately, those lymph nodes which might be the first to be stimulated (from the gluteal, subaortal and rectal area) cannot be removed by the current surgical procedures.

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