AN IMMUNOHISTOLOGICAL STUDY OF CELLS INFILTRATING PROGRESSIVE AND REGRESSIVE TUMORS INDUCED BY TWO VARIANT SUBPOPULATIONS OF A RAT COLON CANCER CELL LINE

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In order to understand the mechanisms leading up to progression or regression, tumors resulting from the s.c. inoculation of progressive or regressive variants of a cell culture established from a chemically-induced rat colonic carcinoma were subjected to sequential histological study. As immunological factors have been previously described in this system of progressive or regressive tumors, special interest was given to inflammatory cells, T and B lymphocytes and macrophages, located inside and outside the tumor. Immunohistological methods using monoclonal or polyclonal antibodies and enzyme histochemistry were performed to identify different populations of infiltrative cells. In both variants of tumors an accumulation of these cells were seen at the periphery of the tumor, surrounding the nodules. In contrast, very few inflammatory cells, macrophages or T lymphocytes were seen inside the clumps of tumor cells where cytotoxic cells could have a contact-dependent tumoricidal effect. Only small differences were found between progressive and regressive tumors in the density of the various populations of T helper, T cytotoxic/suppressor, B lymphocytes or macrophages inside or around the tumor nodules. On the other hand, progressive and regressive tumors clearly differ in the relationship between tumor cells and the fibroblastic reaction they induce. Regressive tumors were rapidly encircled by a fibroblastic reaction isolating them from the peripheral tissues. The fibroblastic reaction was less dense around the progressive tumor cells which were able to migrate and invade the periphery. This suggests that immunological factors leading to tumor progression or regression could act indirectly through a control of the fibroblastic reaction, rather than through a direct cytotoxic effect on the tumor cells.

From a cell culture of a colon carcinoma chemically induced in the rat, 2 variant subpopulations have been isolated; one gives progressive tumors and the other gives tumors which regress in 3 to 5 weeks when inoculated into syngeneic rats (Martin et al., 1983). The role of the immune system in the control of tumor progression or regression was suggested by the progressive growth of the regressive cell line when inoculated into nude mice and by the possibility of transferring tumor resistance or tumor enhancement in a Winn-type assay (Caignard et al., 1985).

In the present study, a sequential histological and immunoenzymatic study of progressive and regressive tumors was carried out in order to identify the cell populations which could play a role in tumor progression or rejection. In spite of the use of multiple markers, no clear-cut difference was found between macrophage and lymphocyte subpopulations infiltrating progressive and regressive tumors. On the other hand, regressive tumors clearly differ from progressive ones in eliciting an early fibroblastic reaction leading to a fibrous encapsulation which could play a role in the mechanisms of tumor regression.

MATERIAL AND METHODS

Animals and tumor cell lines

Syngeneic BDIX strain rats have been bred in our laboratory by brother-sister mating since 1971. Sublines TRb (progressive) and TSb (regressive) were clones isolated from tumor-cell subpopulations originating from a colon carcinoma induced by 1,2-dimethylhydrazine in a BDIX rat (Martin et al., 1983; Caignard et al., 1985). They were cultivated in Ham's F10 medium supplemented with 10% fetal calf serum, then detached by sequential treatment with EDTA and trypsin, washed in complete medium and suspended in Minimum Eagle medium (Gibco, Paisley, Scotland), at a concentration of 2 x 106 cells/ml. In each of 2 groups of 40 rats, 20 animals (10 males and 10 females) were inoculated s.c. in the side of the thoracic wall with 106 TRb or TSb cells. The age of the animals was 7 months in group I and 3 months in group II. In each group, 4 rats inoculated with TRb cells and 4 inoculated with TSb cells were randomly chosen for killing 6, 12, 18, 25 and 30 days after tumor-cell injection. Tumors were weighed and frozen for cryosection in group I or fixed (Gendelman et al., 1983) in group II.

Histological study

A conventional histological study was carried out on the 40 tumors fixed in Gendelman’s solution and embedded in paraffin according to Sainte-Marie (1962). The 3-µm sections were stained with hematoxylin-phloxine-saffron, modified trichrome Masson and periodic-acid Schiff techniques.

An immunoenzymatic study was performed both on cryosections (group I) and on fixed tumors embedded in paraffin (group II). The 2 procedures were complementary since fixation and embedding gave a better conservation of the tissue structure but inhibited immunoenzymatic staining for several of the monoclonal antibodies (MAbs). The slides were stained by the avidin-biotin-peroxidase complex method (Hsu et al., 1981) with modifications (Martin et al., 1986). The 7 murine MAbs used for typing rat lymphocytes and macrophages are listed in Table I. W3/13 and OX8 antibodies were obtained from Seralab (Crawley Down, Sussex, UK), and W3/25 and OX19 antibodies from Serotec (Realeaf, Paris, France), as ascitic fluid at concentrations of 1 or 2 mg/ml. KiM2R MAb was a gift from Prof. H.H. Wacker (Kiel, FRG). B lymphocytes were labelled on fixed and paraffin-embedded sections with a polyclonal rabbit anti-rat IgM antibody (Nord, Tilborg, The Netherlands) diluted 1:50, then the avidin-biotin-peroxidase technique was performed using a goat anti-rabbit IgG biotinylated antibody (Sigma, St. Louis, MO) diluted 1:50 as second reagent. The validity of the immunoenzymatic technique was checked using fixed or fresh frozen sections of rat spleen which contain all the cells studied in our work. In the absence of mouse MAbs against rat lymphocyte or macrophage antigens, only mast cells were stained. Macrophages were also identified on sections of fixed and embedded tumors by their non-specific esterase content, using alpha-naphthol ASD acetate as substrate (Koski et al., 1976).

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The density of inflammatory cells was independently evaluated by 2 observers (M.S.M. and F.M.). Every slide was examined at least twice by each observer, first to compare TRb and TSb tumors of the same age, then to evaluate tumor kinetics. Three areas were distinguished in each tumor for location of the inflammatory cells: the clumps of cancer cells, the fibroblastic and inflammatory corona surrounding them and the connective or muscular tissue at the periphery of the tumor. Due to the heterogeneity of the tumors and variance in the thickness of cryostat sections, no attempt was made to count the cells stained by the different reagents. However, an arbitrary scale graded from 0 to 4 was used to compare the 2 tumors and establish the kinetics of inflammatory cells. This score did not differ by more than one grade when the same slide was read at intervals of several weeks.

Slides were photographed using a Leitz Dialux 20 microscope with a Vario Orthomat apparatus. Additional contrast was achieved by use of a blue glass filter.

RESULTS

Kinetics of growth of TRb and TSb tumors

The growth of the 2 variant tumors, TRb and TSb, reproduces the same sequence of events previously obtained with the same tumors (Martin et al., 1983). Measurable tumors were observed in all inoculated animals. At the end of the experiment, 30 days after inoculation, tumors were found in all 8 animals injected with TRb cells (mean tumor weight: 411 mg), whereas a small tumor (mean weight: 10 mg) was found in only 2 of the 8 rats inoculated with TSb cells. The kinetics of TSb tumor regression was not exactly the same in the two groups, tumors reaching their maximum size at 25 days in group I and at 12 days in group II. This difference was significant (p < 0.001) and could be due to the difference in age between the rats of groups I and II at the time of tumor-cell inoculation.

Conventional morphology

Hematoxylin-phloxin saffron and Masson's trichrome staining of tumors fixed in Gendelman's medium allowed the evolution of progressive (TRb) and regressive (TSb) tumors to be studied.

(i) Progressive tumors (Fig. 1a,c,e,g)

At 6 days after inoculation, TRb cells are organized into cords dispersed in a matrix of collagen fibers developing in the subcutaneous fat tissue or inside the disrupted muscular layers. A heavy fibro-inflammatory cellular reaction circumscribes the tumor nests. At 12 days the tumor comprises several nodules (6 to 10 per section) in which tumor cords are centered on an acellular area of connective tissue and are surrounded on their external side by a thick fibro-inflammation cellular layer. At 18 days, the tumor is larger but maintains the same organization. At 25 days, the fibro-hyaline center of each nodule is surrounded by a scalloped ring of tumor cords separated from the thin peripheral fibro-inflammatory ring by a moderate infiltrate of mononuclear cells. At 30 days, tumor cells are intermingled with mononuclear cells and fibroblasts which surround and infiltrate the tumor. Areas of necrosis and fibrosis are found in the center of the tumors.

(ii) Regressive tumors (Fig. 1b,d,f,h)

At 6 days after inoculation, TSb cells are organized into clumps encircled by fibroblasts and mononuclear cells. At this stage, TSb tumors differ from TRb tumors by their higher fibroblast content. At 12 days, the tumor clumps, thicker and more crowded than in TRb tumors, are almost confluent but include central necrotic areas and a peripheral fibro-inflammatory stroma. They are separated from the non-invaded tissue by a thick layer of fibroblasts extending in fibrous septa between the nodules. At 18 days, the cancer cells are exclusively located at the center of the tumor nodules, different from progressive tumors whose TRb cancer cells are found at the periphery. The TSb tumors are still crowded but scattered areas of necrosis are found; they are still surrounded by a thick layer of fibroblasts and mononuclear cells. At 25 days, tumors contain no cancer cells, or only altered cancer cells intermixed with necrosis, epithelioid cells and multinucleated giant cells, scattered among collagen fibers, fibroblasts and inflammatory cells. At 30 days, there was no tumor in 3 rats; in the 4th animal, there was a fibrous granuloma containing some giant cells but no tumor cells.

Immunoenzymatic staining

Polyclonal anti-IgM serum, only used on fixed paraffin sections, stained the membrane of B lymphocytes. In both progressive and regressive tumors, B lymphocytes were absent at 6 days after inoculation. At 12 days, a few cells were labelled in the lumen of the vessels. A more and more heavy infiltration by B lymphocytes was found from 18 to 30 days after inoculation in the corona of inflammatory cells surrounding the tumors, but B lymphocytes were never found inside the nests of tumor cells. No difference in IgM+ cell density or location was found between progressive and regressive tumors.

The kinetics of labelled inflammatory cells with MAbs on frozen sections is summarized in Table II.

W3/13 and OX 19 MAbs, were used as markers of T lymphocytes. On frozen sections both sera labelled the membrane of lymphoid cells at a similar location; but W3/13 serum can also be used on fixed paraffin sections. At 6 and 12 days after cell inoculation, W3/13 and OX 19+ lymphocytes were almost exclusively located in the corona of inflammatory cells surrounding the TRb and TSb tumors and in the fatty and muscular tissues at the periphery of the tumors (Fig. 2). The infiltration of T lymphocytes into the corona increased with
time, without any difference between progressive and regressive tumors. In the TSb tumors only, a few W3/13+ lymphoid cells were found inside the tumor clumps, in direct contact with the tumor cells, from the 6th day after inoculation. From the 12th day, they were occasionally observed in both tumors. A few W3/13+ polymorphs, recognizable by their nucleus, were found in necrotic areas or at the periphery of the tumors.

**TABLE II - IMMUNOENZYMATIC STAINING OF INFLAMMATORY CELLS BY MONOCLONAL ANTIBODIES**

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<tr>
<th>Antibody</th>
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<th>TSB 6</th>
<th>TRb 12</th>
<th>TSB 12</th>
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<th>TRb 25</th>
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**B - Cells infiltrating the tumor clumps**

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<th>TSB 6</th>
<th>TRb 12</th>
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1 Days after tumor cell inoculation.

Semi-quantitative data represent the estimate of the number of cells labelled by the different MAbs (arbitrary scale graded from 0 to + + + +). Each result is the mean of 4 tumor estimates except for tumor TSb at 30 days where only one rat bore a tumor. Results are given separately for the center of the tumor and its periphery corresponding to the corona of inflammatory cells surrounding the tumor. Only the results obtained on fresh-frozen sections have been reported on this Table.

**Enzymatic staining**

Non-specific esterase histochemical staining was performed on fixed paraffin sections. It labelled mainly macrophages, both in peripheral tissues and in the corona of inflammatory cells. Infiltration increased with time and was particularly dense in regressing TSb tumors. KiM2R+ cells found in the clumps of tumor cells were also stained for esterase. Furthermore, esterase staining labelled the cytoplasm of small mononuclear cells, presumably monocytes, surrounding TRb tumors.
and TSb tumors 6 days after inoculation. This staining was diffuse and distinct from the dot-like granular staining observed in the cytoplasm of some T lymphocytes. Giant cells, observed after regression of TSb tumors, were not labelled or only faintly labelled for esterase, although they probably originated from esterase-positive tumor-infiltrating macrophages.

**DISCUSSION**

TRb and TSb cells are 2 variant subpopulations of cancer cells originating from the same tumor and differing in their tumorigenic potential when inoculated into syngeneic animals. A comparative and sequential study of the progressive tumors induced by TRb cells and the regressive tumors induced by TSb cells was carried out to obtain information on the mechanisms leading to tumor progression or regression. Our study included the identification of macrophage and lymphocyte subpopulations since these cells may play a role in an immune mechanism controlling tumorigenicity. That the immune system intervenes in the difference in growth properties found between TRb and TSb tumor cells has been previously established (Caingard et al., 1985). TSb cells, which only give regressive tumors in the syngeneic BDIX rat, grow as progressive tumors when inoculated into thymus-deficient nude mice. Regressive TSb tumors are able to immunize syngeneic BDIX rats against progressive TRb tumors and spleen lymphocytes taken after TSb tumor rejection inhibit the growth of progressive tumors when inoculated with TRb cells in a Winn-type transfer assay. TSb cells produce progressive tumors in BDIX rats bearing an established TRb tumor or when they are inoculated together with spleen cells from rats bearing a large TRb tumor. These observations suggest that TSb cells induce an immune response against both TRb and TSb cells and that TRb tumors are able to suppress this immune response.

It could have been expected that the difference in immune response induced by TRb and TSb cells would be reflected by a clear-cut difference in the density and/or class of inflammatory cells infiltrating the 2 varieties of tumor. These differences should have been found especially in the cells in direct contact with the tumor cells, since the best known immune mechanisms leading to tumor cell destruction require a direct contact between the target tumor cell and the effector cell. Cytotoxic T cells, NK cells or macrophages. In fact, only faint differences were observed. In both tumors, a progressive accumulation of macrophages, T helper and cytotoxic/suppressor lymphocytes and B lymphocytes was seen in muscular and connective tissues peripheral to the tumor and in a corona of inflammatory cells surrounding the tumor cells. Most of the cells found in these locations were not in direct contact with the cancer cells and could only indirectly interfere with them. On the other hand, only a few inflammatory cells were found inside the clumps of cancer cells, where a contact-dependent cytotoxic effect was possible. These inflammatory cells belong to 2 distinct cell populations. A minority of these cells were small lymphoid cells with membrane labelling by W3/13 and OX 8 antibodies, but were not labelled by W3/25 or OX 19 antibodies. They could be activated suppressor or cytotoxic T cells or NK cells since OX 19 antibody does not react with these 2 varieties of cells (Oettingen and Vollmer, 1986). The second population of cells infiltrating the clumps of tumor cells were large polygonal cells, sometimes with dendritic extensions interspersed between the tumor cells. They can be classified as macrophages since they are labelled by the macrophage-specific KiM2R MAb and are diffusely stained by esterase reagent. However, these KiM2R+, OX 8+, W3/25- macrophages are phenotypically distinct from the KiM2R+, OX 8+, W2/25+ macrophages found outside tumor cell clumps. To our knowledge, reactivity of rat macrophages with OX 8 MAb has not been previously reported, but Mason et al. (1983) incidentally noted that human splenic macrophages react with OKT8 antibody, a marker of cytotoxic/suppressor T cells which can be considered as a human equivalent of OX 8 antibody. We do not know whether OX 8+ lymphocytes and macrophages share a common antigen or different antigens with a common epitope recognized by OX 8 MAb. Infiltration of tumor cell clumps by OX 8+ lymphoid cells and macrophages is common to the progressive TRb tumors and the regressive TSb tumors when studied 12 to 25 days after tumor cell inoculation. However, these cells were absent in the clumps of TRb tumor cells 6 days after inoculation, whereas TSb cell clumps were infiltrated by OX 8+ inflammatory cells, predominantly macrophages, at this stage. With the exception of this time-restricted difference, our data do not support a direct cytotoxic role of inflammatory cells as the explanation of the difference in tumorigenicity between TRb and TSb tumor cells.

Vollmer et al. (1986) have recently compared infiltration by inflammatory cells, labelled with OX 8, W3/25 or KiM2R antibodies, in progressive or regressive tumors obtained by inoculating various amounts of mammary carcinoma cells to syngeneic rats. As in our work, they found only marginal differences between progressive and regressive tumors in their infiltration by mononuclear cells. In disagreement with our results, Vollmer et al. found that the cells in direct contact with the carcinoma cells were W3/25+, whereas OX 8+ cells were confined to the periphery of the tumor. However, OX 8+ cells were seen among carcinoma cells in tumors undergoing regressive change. In contrast to these minor differences, Ishii et al. (1984) and Ibayashi et al. (1985) found large differences. Regressive tumors resulting from a specific immunization preceding tumor cell challenge in rats differed from progressive ones by a massive accumulation of OX 8+ lymphocytes.
phoid cells, presumably cytotoxic T lymphocytes, whereas macrophages are only a minor component of the inflammatory cell reaction. It is possible that specific immunization preceding tumor cell inoculation explains such differences between the results obtained by the Japanese investigators and those reported here concerning tumor infiltration by OX 8+ cells.

If minor differences only are found between progressive TRb tumors and regressive TSb tumors in the varieties of inflammatory cells infiltrating them, both tumors clearly differ in the relation between tumor cells and connective tissue. As early as 6 days after tumor cell inoculation, TRb tumor cells grow in a matrix of loose connective tissue containing collagen fibers but few fibroblasts, whereas TSb cell cords are surrounded by several concentric layers of fibroblasts. From the 12th day onwards, fibroblasts form a thick shell encircling the clumps of TSb tumor cells, isolating them from each other and from the surrounding tissues. This fibroblastic shell is not penetrated by isolated TSb cells. On the other hand, TRb cells seem to be able to invade the looser connective tissue which surrounds them and migrate towards the peripheral area. In this outward migration, they leave an acellular central area of collagen-rich connective tissue.

Similar differences between progressive and regressive tumors have been previously reported by Key and Haskell (1981) in a model of transplanted murine mammary carcinoma. These authors linked the progression of the tumors with the capacity of the tumor cells to invade the capsule surrounding them, whereas the regressive tumors were completely encapsulated in a dense fibroblastic shell restraining their progression.

In our model, regression of the TSb tumors could be due to the fibroblastic capsule which forms a physical barrier to tumor progression but also limits the nutritional supply to the tumor cells. The progression of TRb tumors could be due to the ability of these cells to induce a poor fibroblastic reaction or to their capacity to destroy the connective tissue through specific enzymes (reviewed in Van den Hooff, 1986). This mechanistic conception of the difference between progressive and regressive tumors has to be reconciled with the above-mentioned data suggesting a major role of the immune system in tumor progression or regression. Mononuclear cells intervening in the immune response, macrophages and T lymphocytes, are able to elaborate soluble factors regulating fibroblast proliferation and collagen synthesis (reviewed in Freundlich et al., 1986). It is possible that the differential immune responses induced by TRb and TSb cells (Caignard et al., 1985) are mostly efficient on tumor progression or regression through their interaction with fibroblasts. Lymphocytes, macrophages, fibroblasts and tumor cells could constitute an integrated system (Van den Hooff, 1986) which should be considered as a whole in attempting to understand the mechanisms leading to tumor progression or regression. The effects of TRb and TSb tumor cells on fibrin deposit and fibroblast growth, either directly or through immunological factors, have to be investigated to understand the mechanisms leading to tumor progression or regression.

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