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Expression of the Monoclonal Antibody-Defined CAR-3 Epitope on Neoplastic and Preneoplastic Lesions of the Colon Mucosa*

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Abstract—*The AR-3 monoclonal antibody, which defines the tumor-associated antigen CAR-3, was previously found to be able to discriminate between neoplastic cells in gastric, pancreatic, colonic, ovarian and endometrial carcinomas and their normal counterparts. In fact, it strongly reacts with carcinomatous cells at the level of both the glycocalix and the cytoplasm, while its reactivity with normal tisues is restricted to the glycocalix of few mucin-producing epithelial cells. We have now investigated the reactivity of this antibody with immunohistochemical techniques on a series of formalin-fixed paraffin-embedded specimens, from precancerous and cancerous lesions of the large bowel which were classified as adenomas with mild, moderate or severe dysplasia, adenomas with cancer and adenocarcinomas, respectively. It was found that the intensity and extent of the staining correlated with the degree of dysplasia and that the highest expression of the CAR-3 epitope was detectable in adenocarcinomas. Also the localization of the staining in the lesions displayed an increasingly complex pattern, going from linear in adenomas with mild dysplasia to a very strong intracytoplasmic and/or intraluminal expression in adenomas with severe dysplasia or adenocarcinomas.*

INTRODUCTION

IT IS generally accepted that adenomas are the most frequent precancerous lesions of the large bowel [1–3], their malignant potential varying with size, histological type, degree of dysplasia and age as well as a variety of other factors [4–9]. Diagnostic problems may arise in the evaluation of the malignant potential of a lesion, since the epithelial abnormalities observed encompass a morphological continuum with an increasing degree of dysplasia, which makes it difficult to assign them precisely to one of the four proposed groups (adenomas with mild, moderate, severe dysplasia and adenocarcinomas [2]). An additional problem is the fact that, in some adenomas, areas with different degrees of dysplasia may coexist [6]. In this progression, intraepithelial carcinoma and intramucosal carcinoma represent the next two steps, where carcinomatous cells are localized in foci restricted to the original crypts of Lieberkuhn or already invade the lamina propria, respectively [5]. Finally, the unequivocal sign of malignant transformation is considered to be the invasion of the muscularis mucosae [1, 6].

From the above description it is clear that the localization of tumor cells is extremely important for diagnosis and subsequent therapy. An additional problem arising in this context is the fact that adenomas may be traumatized during the excision and microscopic foci of pseudo-invasion of the stalk may appear [7, 10]. Moreover the incorrect handling of specimens (orientation and sectioning) may give rise to artefacts, false images, pseudocarcinomatous appearance. Finally, in a few cases, it may be necessary to examine numerous serial sections, in order to ascertain the invasive nature of the carcinoma and to make a definite diagnosis. It is thus clear that a diagnosis based only on morphological criteria may sometimes be difficult and other independent criteria will be helpful to the pathologist. In this context, antigenic [11–13] and/or

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biochemical modifications [14–16] have been reported to be associated with the neoplastic transformation, and MAbs defining these qualitative or quantitative alterations could represent specific and objective tools in cancer diagnosis. Moreover, the identification of selective markers evaluating the malignant potential of adenomas could also be extremely important. In the laboratory of some of us (M.P., P.M.C.), a monoclonal antibody (MAb), identified as AR-3, has been developed and proven capable of discriminating between pancreatic, gastric, colonic, ovarian and endometrial carcinomas and their corresponding normal tissues, when assayed on formalin-fixed paraffin-embedded sections by the avidin–biotin–peroxidase method [17]. Among the normal tissues screened, staining of only some normal mucin-producing epithelial cells was occasionally observed. The AR-3 MAb-defined epitope was found to be different from the MAb-defined tumoral markers described so far and, on the basis of its tissue distribution, it was called CAR-3. CAR-3 is expressed on a heavily glycosylated cellular component displaying a molecular weight > 400 kd, whose antigenic activity is sensitive to metaperiodate oxidation (manuscript in preparation).

In the work reported here, the AR-3 MAb was tested by immunohistochemical techniques on sections of different colorectal adenomas and carcinomas, to analyze whether any correlation could be observed between the degree of dysplasia present in the neoplastic lesion and the expression of the CAR-3 epitope.

MATERIALS AND METHODS

Production of the monoclonal antibody

The methodology used for the generation and characterization of the AR-3 MAb was reported in detail previously [18, 19]. Briefly, it was selected from a hybridoma culture obtained after fusing the non-producing variant myeloma cell line P3.X63.Ag8.653 with splenic lymphocytes from BALB/c mice hyperimmunized with paraformaldehyde-fixed A431 epidermoid carcinoma cells. The hybrid was cloned twice by limiting dilution and adapted for growth in an ascitic form in pristane-primed BALB/c mice.

Histological specimens

Immunoperoxidase and immunofluorescence staining with the AR-3 MAb was performed on 73 formalin-fixed paraffin-embedded biopsies of the large bowel. A total of 52 adenomas was examined; of these some were prepared from colectomy specimens and some from polypectomies. Histological type was classified into tubular, tubular-villous and villous adenoma, respectively [2]. In grading dysplasias as mild, moderate or severe, the criteria proposed by the World Health Organization in 1976 [20] and by Konishi and Morson [6] were followed. The WHO nomenclature of well, moderately and poorly differentiated carcinomas is used in this paper [20]. Twenty-one cases of adenocarcinoma were also examined.

Immunoperoxidase (ABC method)

Tissue sections were deparaffinized and hydrated through xylene and graded alcohol series, then washed in phosphate buffered saline (PBS) pH 7.2. The endogenous peroxidase was blocked by treatment with hydrogen peroxide, followed by the periodic acid–sodium borohydride sequence according to Heyderman and Neville [21]. Slides were then sequentially incubated at room temperature with normal horse serum diluted in PBS–azide–0.1% bovine serum albumin (BSA) for 20 min and then with the AR-3 MAb diluted 1 : 10 for 4 hr in a moist chamber. Sections were incubated with biotinylated horse antibodies directed against mouse immunoglobulins (Vetor Lab., Burlingame) for 30 min and then washed in 0.1 M carbonate buffer pH 9.5 and incubated for 45 min with the ABC complex (Vector Lab., Burlingame), prepared just before use in carbonate buffer, according to Bussolati and Gugliotta [22]. Negative controls included samples where the AR-3 MAb was replaced by non-immune mouse serum or immunoglobulins. The peroxidase reaction was developed with diaminobenzidine tetrahydrochloride or 3-amino-9-ethyl-carbazole. Sections were counterstained with Mayer's haemalum, mounted and read in a double blind fashion.

Immunofluorescence

Slides were deparaffinized, hydrated, washed in PBS and incubated sequentially in a moist chamber for 20 min at room temperature with normal goat serum diluted 1 : 10 in PBS–azide–BSA and for 4 hr with the AR-3 MAb diluted 1 : 10. Sections were incubated for 30 min with biotinylated goat antibodies directed against mouse immunoglobulins (Tago, Burlingame) diluted 1 : 20. After washing in carbonate buffer pH 9.5, slides were incubated for 45 min in a moist chamber with a 0.003% solution of fluorescein-isothiocyanate-labelled Avidin (Sigma, St. Louis) in carbonate buffer. Sections were mounted in buffer–glycerol. As in the case of the ABC method, negative controls included samples where the AR-3 MAb was replaced by non-immune mouse serum or immunoglobulins and specimens were read in double blind fashion.

RESULTS

Preliminary experiments were performed to assess the specificity of the AR-3 MAb on specimens derived from normal and neoplastic tissues from different organs and the results previously obtained in another laboratory [17] were confirmed. Moreover, in some experiments, different dilutions of the AR-3 MAb were tested, in order to define optimal usage conditions. Dilutions of 1/10–1/20 proved to be the best, with an end-point dilution of 1/80. A 1/10 dilution was chosen for the present study, since this concentration had already been used in previous work with this monoclonal antibody [17].

In this study, formalin-fixed paraffin-embedded sections derived from 73 colorectal excisional biopsies were examined for their reactivity with the AR-3 MAb using the immunoperoxidase (ABC) and immunofluorescence (IF) techniques. Comparable results were obtained with the two techniques. In Table 1, the results obtained with the ABC method are reported and the intensity, extent and localization of staining in each of the four groups of lesions examined is described. Areas displaying the highest degree of atypia were selected in all cases. The adenomas studied were classified as adenomas with mild, moderate or severe dysplasia, respectively, the last group also including adenomas with intraepithelial carcinomas. The fourth group comprised invasive carcinomas in adenomas and adenocarcinomas, which were further classified as poorly, moderately or well differentiated. Staining intensity was graded as weak (+), moderate (++) or strong (+++). Staining extent was schematically scored as ± (focal positive) in those cases in which < 1% of the section was stained, as + or as ++, when < 50% or > 50% of the tissue lesion was stained, respectively.

Colorectal adenomas

In the first group, which comprised 15 cases of adenoma showing mild dysplasia, 4 cases (27%, Fig. 1) were negative, 8 cases (53%) were weakly stained (Figs. 3C, D) and 3 (20%) were moderately stained. No case displayed strong positivity. When staining extent was considered, 7 cases (47%, Fig. 2) displayed < 50% tissue lesion stained, only 1 case (6%) > 50%, and 7 cases (47%) were negative or focally positive.

In the second group (21 adenomas with moderate dysplasia), 7 cases (33%) were negative, 5 (24%) were weakly stained, 5 (24%) were stained with a moderate intensity and 4 cases (19%) were strongly decorated. As regards to staining extent, 6 cases (29%) were stained in < 50% of the lesions, 8 cases (38%) were focally positive and 7 (33%) were negative. In the lesions with both mild and moderate dysplasia, the AR-3 MAb was mostly reactive with the surface of epithelial cells on the outer mucosal layer (Figs. 3C, D, E). This linear pattern of reactivity was the result of the staining of the apical cellular pole. Diffuse cytoplasmic positivity was sometimes detected and in few cases some deposits of secreted material within the pseudotubules were also decorated (Table 1; Figs. 3E, F).

In the third group, 10 adenomas with areas of severe dysplasia and 2 adenomas with intraepithelial carcinoma were examined. Three cases (25%) were negative, 3 (25%) were weakly positive, 2 (17%) were moderately stained and finally 4 cases (33%) revealed strong positivity. When taking into account staining extent, 3 cases (25%) were negative, 3 (25%) were focally positive, 4 (33%) and 2 (17%) cases stained respectively less or more than 50% of the lesion.

It is known that areas with different degrees of atypia may coexist in adenomas. In these cases we have generally detected a predominant CAR-3 localization in severely dysplastic or carcinomatous areas. Only few cases failed to show this correlation. Positivity was localized both at the apical region of the cell and in the cytoplasm, in the latter case being more abundant (Fig. 4A); deposits of exfoliated and/or secreted material were also stained within the pseudotubules.

In the three groups of adenomas examined, the CAR-3 epitope did not show a preferential association with any particular histological type (tubular, tubular-villous or villous).

Normal mucosa was examined when section orientation allowed it. Sometimes some normal mucosa cells near the lesion were stained (Figs. 3A, B). The apical border of cells lining the glandular lumen and amorphous debris close to the superficial epithelium were reactive. In a few cases intracytoplasmic reactivity was also observed.

Adenocarcinomas

Twenty-five cases of adenocarcinomas of the large bowel including 4 cases of adenomas with invasive carcinomas were examined. The AR-3 MAb revealed strong positivity in 14 cases (56%) and moderate reactivity in 4 cases (16%); 3 cases were weakly stained (12%) and 4 cases (16%) were negative. In 9 cases (36%) the reactivity was observed in > 50% of the tissue lesion, in 7 cases (28%) in < 50%; focal positivity was observed in 5 cases (20%), negativity in 4 cases (16%) or focally positive. Besides intense intracytoplasmic positivity (Figs. 4B, D), large intraluminal deposits within the lamina propria and submucosal connective tissue were detected (Table 1; Fig. 4C).

DISCUSSION

For diagnostic, therapeutic and prognostic purposes, it is imperative to define simple objective

Table 1. *Reactivity of the AR-3 monoclonal antibody with adenomas and adenocarcinomas of the large bowel*

Case No.	Histological type	Reactivity		Pattern of labelling		
		Extent	Intensity	Linear	Cytoplasmic	Intralum.
I. Adenomas with mild dysplasia						
1	Tubular	−	−	−	−	−
2	Tubular	−	−	−	−	−
3	Tubular	−	−	−	−	−
4	Tubular	−	−	−	−	−
5	Tubular	±	+	+	−	−
6	Tubular	±	+	+	−	−
7	Tubular	±	+	+	−	−
8	Tubular	+	+	+	−	−
9	Tubular	+	+	+	−	−
10	Tubular	+	+	+	−	−
11	Tubular	+	++	+	+	−
12	Tubular	+	++	+	−	−
13	Tubular-villous	+	+	+	−	−
14	Tubular-villous	++	+	+	−	−
15	Tubular-villous	+	++	+	−	−
II. Adenomas with moderate dysplasia						
1	Tubular	−	−	−	−	−
2	Tubular	−	−	−	−	−
3	Tubular	−	−	−	−	−
4	Tubular	−	−	−	−	−
5	Tubular	+	+	+	−	−
6	Tubular	+	+	+	−	−
7	Tubular	+	+	−	+	−
8	Tubular	+	+	+	−	−
9	Tubular	+	++	+	+	+
10	Tubular	+	++	+	−	−
11	Tubular	+	++	+	+	−
12	Tubular	+	+++	+	+	−
13	Tubular	+	+++	+	−	−
14	Tubular-villous	−	−	−	−	−
15	Tubular-villous	−	−	−	−	−
16	Tubular-villous	−	−	−	−	−
17	Tubular-villous	±	++	−	+	+
18	Tubular-villous	+	+++	−	+	+
19	Tubular-villous	±	+++	+	+	+
20	Villous	±	+	+	−	−
21	Villous	±	++	−	+	−
IIIa. Adenomas with severe dysplasia						
1	Tubular	−	−	−	−	−
2	Tubular	±	+	−	+	−
3	Tubular	+	+++	+	+	+
4	Tubular-villous	−	−	−	−	−
5	Tubular-villous	±	++	+	+	−
6	Tubular-villous	+	++	+	+	−
7	Tubular-villous	++	+++	+	+	+
8	Tubular-villous	++	+++	+	+	−
9	Villous	−	−	−	−	−
10	Villous	+	+	+	+	−
IIIb. Adenomas with intraepithelial carcinoma						
11	Tubular-villous	±	+	+	+	+
12	Tubular-villous	+	+++	+	+	+
IVa. Adenomas with invasive carcinoma						
1	Tubular	±	+	+	−	+
2	Tubular	+	+++	+	−	+
3	Tubular	++	+++	+	+	+
4	Tubular-villous	++	+++	−	+	−

Case No.	Histological differentiation	Reactivity		Pattern of labelling		
		Extent	Intensity	Linear	Cytoplasmic	Intralum.
IVb. Adenocarcinomas						
5	Poor	+	+++	+	+	−
6	Moderate	−	−	−	−	−
7	Moderate	−	−	−	−	−
8	Moderate	−	−	−	−	−
9	Moderate	−	−	−	−	−
10	Moderate	±	+	−	+	−
11	Moderate	±	++	−	+	−
12	Moderate	±	++	+	−	+
13	Moderate	±	+++	+	−	+
14	Moderate	+	+	−	+	−
15	Moderate	+	++	−	+	+
16	Moderate	+	++	−	+	+
17	Moderate	+	+++	+	+	+
18	Moderate	++	+++	+	−	+
19	Moderate	++	+++	−	+	−
20	Moderate	++	+++	+	+	+
21	Moderate	++	+++	+	+	−
22	Moderate	++	+++	+	+	+
23	Moderate	++	+++	−	+	+
24	Moderate	++	+++	−	+	−
25	Good	+	+++	−	+	+

Staining extent: − negative; ± focal positivity, i.e. < 1% positive tissue section; + < 50% positive tissue lesion; ++ > 50% positive tissue lesion.
Staining intensity: − negative; + weak; ++ moderate; +++ strong.
Pattern of labelling: Intralum. Intraluminal; − absent; + present.

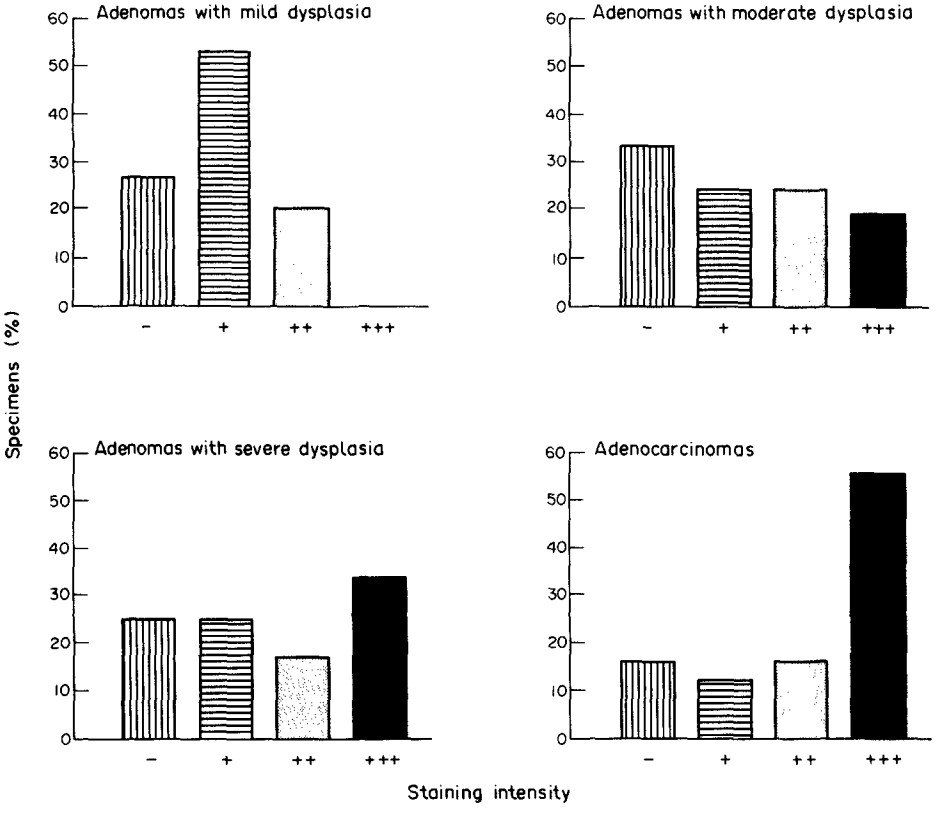

Fig. 1. CAR-3 distribution in preneoplastic and neoplastic lesions of the large bowel. Staining intensity was arbitrarily scored as negative (−), weak (+), moderate (++) or strong (+++). For further details, see Results.

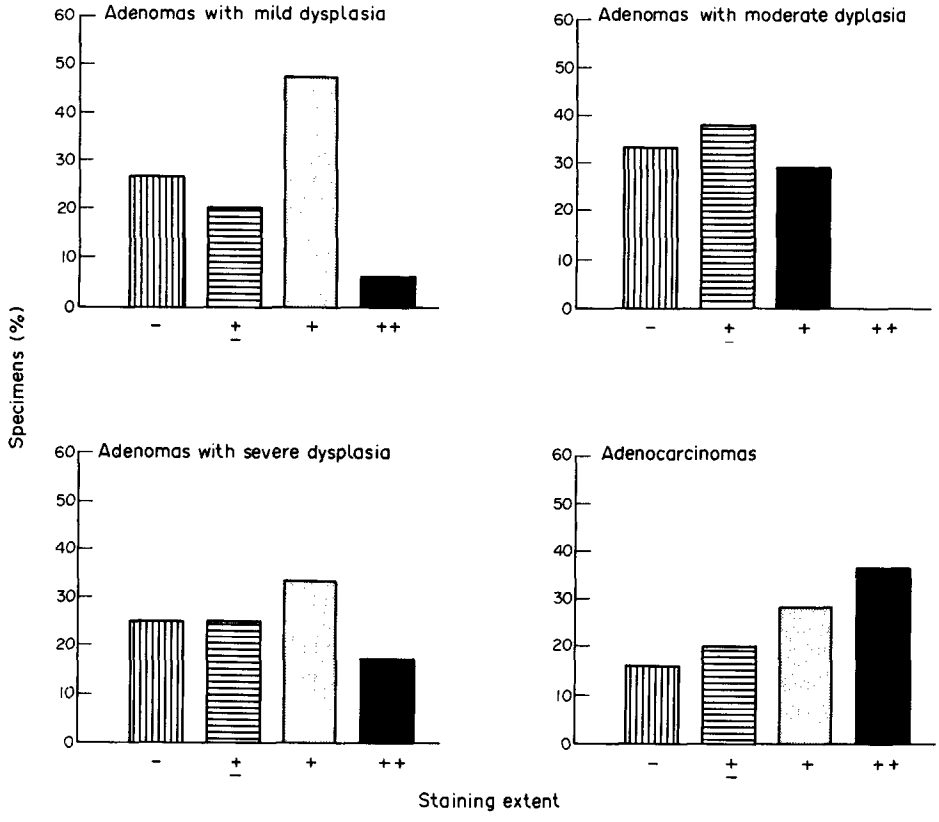

Fig. 2. CAR-3 distribution in preneoplastic and neoplastic lesions of the large bowel. Staining extent was scored as negative (−), focal (±, i.e. < 1% of the section stained), positive (+, i.e. < 50% of the tissue lesion stained), strongly positive (++, i.e. > 50% of the tissue lesion was stained). For further details, see Results.

criteria for the pathological evaluation of adenomas of the gastrointestinal tract.

Since diagnosis based on morphological criteria may sometimes be subjective and not definitive, it is important to use other available parameters to support the conventional histological examination. The identification of tumor associated markers [13], which are the result of qualitative and/or quantitative biochemical changes occurring in neoplastic lesions, has received new impetus from the technology of monoclonal antibodies, which allows the dissection of single antigenic specificities among the complex antigenic pattern of neoplastic cells. MAbs defining tumor associated markers could thus represent a powerful and objective tool for the pathologist and may be helpful in defining the possible neoplastic potential of precancerous lesions.

In this paper we have tested the possibility that the expression of one such tumor-associated marker correlates with the different degree of dysplasia in precancerous lesions of the large bowel. The AR-3 monoclonal antibody was chosen for this study, since it was found capable of discriminating between neoplastic cells in pancreatic, gastric, colonic, ovarian and endometrial carcinomas and their normal counterparts [17]. In fact, the antibody reacted strongly with carcinomatous cells in the above tumors, displaying only a marginal reactivity with a few mucin-producing epithelial cells. Furthermore, the localization of reactivity was also different in the two cases. It was restricted to the glycocalix in the case of normal epithelial cells, while in the case of carcinomatous cells, the cytoplasm was also deeply stained.

The data reported show that the reactivity of the AR-3 monoclonal antibody on the four groups of lesions examined, encompassing adenomas with mild, moderate, severe dysplasia and adenocarcinomas, respectively, varied. This difference was largely quantitative in nature. In fact, adenomas with mild dysplasia showed some reactivity, but only of weak or medium intensity. The extent and intensity of the staining increased parallel to the degree of dysplasia with the highest expression of CAR-3 in the adenocarcinoma group. Moreover, from a morphological point of view, CAR-3 localization in the lesion also showed different patterns, going gradually from a linear expression in adenomas with mild dysplasia to very intense cytoplasmic and/or intraluminal localization in adenomas with cancer and adenocarcinomas. It can thus be concluded that, when positivity to the AR-3 antibody is present, its qualitative evaluation parallels the degree of the dysplasia. In this context, in line

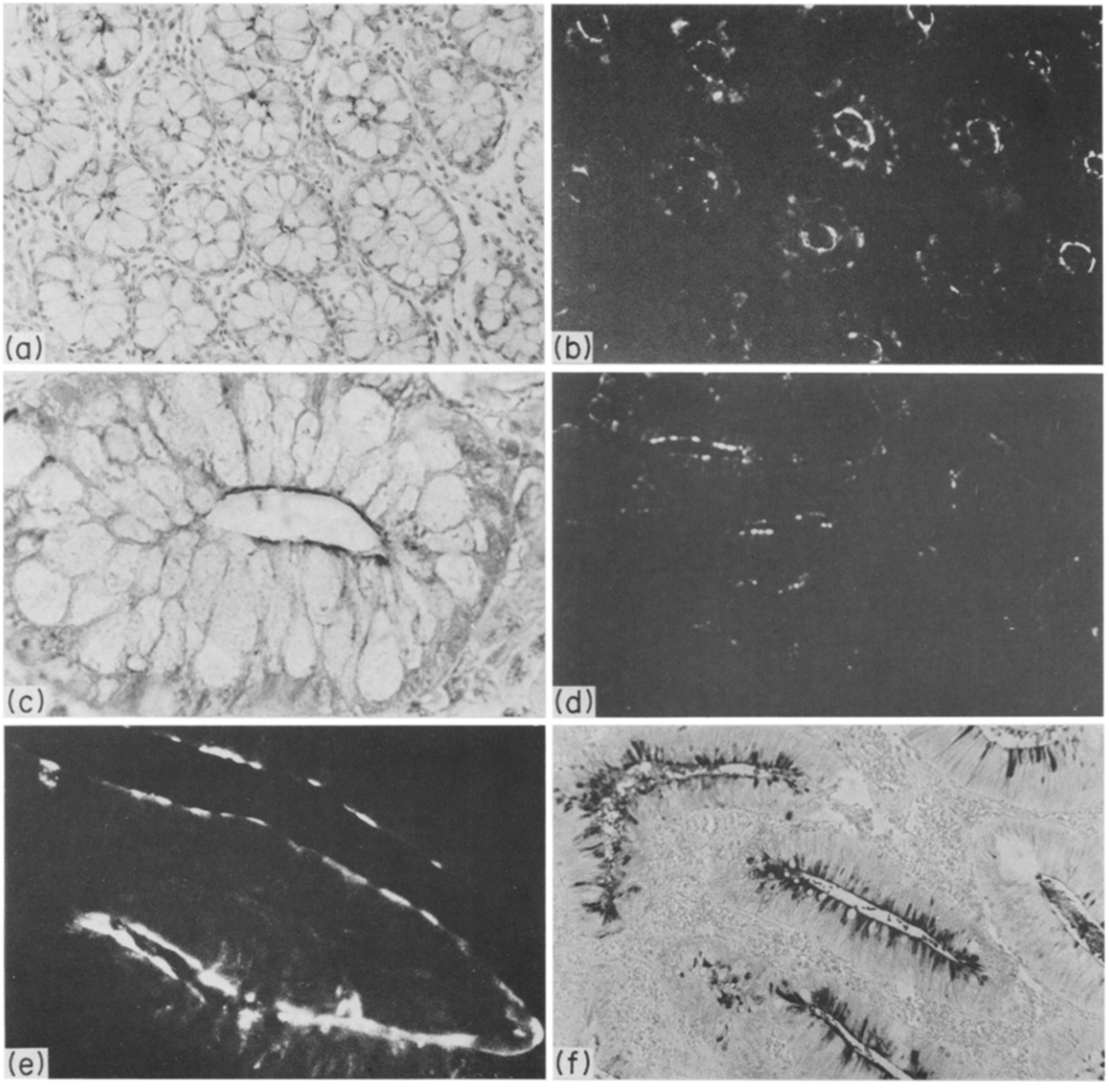

Fig. 3. Human tissues routinely fixed and paraffin-embedded. Sections were stained with AR-3 MAb using either the immunoperoxidase or the immunofluorescence technique. (A) Normal mucosa close to a tubular adenoma with mild dysplasia. Weak staining of the apical pole of the cells (ABC; 183×). (B) Normal mucosa adjacent to adenocarcinoma. Linear staining of the apical border of the cells and some intracytoplasmic spots of positivity. (IF; 183×). (C) Adenoma with mild dysplasia. Weak staining with AR-3 MAb in immunoperoxidase (ABC; 292×). (D) Adenoma with mild dysplasia. Linear staining of the apical cellular pole (IF; 183×). (E) Intense AR-3 staining of the apical surface of cells in adenoma with moderate dysplasia (IF; 292×). (F) Adenoma with moderate dysplasia showing positive intraluminal deposits (ABC; 183×).

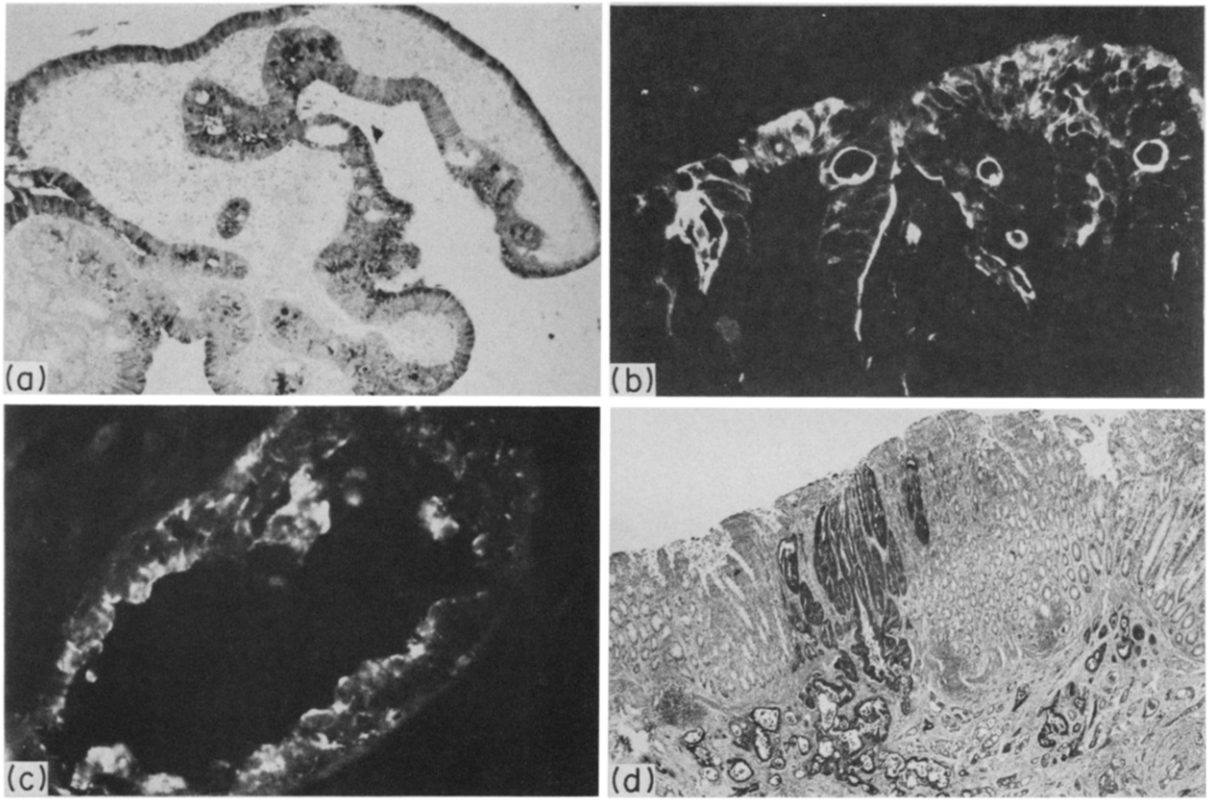

Fig. 4. Human tissues routinely fixed and paraffin-embedded. Sections were stained with AR-3 MAb using either the immunoperoxidase or the immunofluorescence technique. (A) Adenoma with areas of moderate and severe dysplasia. Strong positivity in the cytoplasm of adenomatous cells with the AR-3 MAb (ABC; 24×). (B) Localization of the positivity on the apical pole and in the cytoplasm of adenocarcinomatous cells (IF; 73×). (C) Adenocarcinoma. Intraluminal material stained by the monclonal antibody (IF; 292×). (D) Intense staining of colonic adenocarinoma glands infiltrating normal mucosa (ABC; 18×).

with epidemiological studies, mild dysplasias, which are considered low-risk lesions, expressed low, if any, amounts of CAR-3 epitope, while the other higher risk lesions expressed it in greater amounts.

The positivity of normal mucosa adjacent to adenomas or to adenocarcinomas of the large bowel is not surprising. Biochemical and ultrastructural modifications of this mucosa, which has been defined as "transitional mucosa" when compared to normal mucosa of the colon have been described [14, 16, 23, 24]. We cannot establish whether the positivity of the mucosa adjacent to the lesion should be considered reactive and thus secondary rather than primary and, therefore, indicative of a preneoplastic condition. Moreover, in this context, the possibility that the reactivity was due to the presence of CAR-3 containing material released from the adjacent neoplastic cells and adsorbed onto the normal mucosa cannot be discarded. A similar finding has also been reported for the carcinoembryonic antigen (CEA [1, 12]) and the gastro-intestinal carcinoma antigen (GICA [26]), the most widely studied markers for gastro-intestinal tumors [11, 12, 27–31].

The expression of CEA in preneoplastic lesions of the gastro-intestinal tract has been investigated using immunohistochemical techniques [12, 28, 32–34]. Its usefulness for the evaluation of lesions of the large bowel, however, is still debated, since contradictory results have been obtained in different laboratories. In fact, while some researchers reported a positive correlation between the expression of CEA staining and the degree of dysplasia [34], these findings were not confirmed by others [35]. Moreover, CEA appears to be expressed more frequently in tubular rather than in villous adenomas [33]: ths observation is in contrast with epidemiological studies showing that the latter are at higher risk [2]. In contrast, the expression of CAR-3 in colo-rectal adenomas did not display a preferential association with any particular histological type.

Recently the expression of GICA in dysplasias and adenocarcinomas from 13 patients with chronic ulcerative colitis has been examined using immunohistochemical techniques and a good correlation was found between the presence of GICA and the degree of dysplasia [26]. Anti-GICA monoclonal antibodies were able to discriminate between preneoplastic lesions and regenerative epithelial changes. No correlation was observed, however, between the pattern and extent of staining with the antibody and the degree of dysplasia and, moreover, in two cases of epithelial changes believed to represent a non-neoplastic reaction to inflammation, GICA was found detectable.

From the studies reported here and in the literature, it is clear that neither AR-3 nor anti-CEA or anti-GICA antibodies can trace the totality of adenomas with severe dysplasia, adenomas with cancer or carcinomas of the large bowel, nor can any one of them detect all the neoplastic cells within a malignant lesion. It is therefore suggested that the combined use of all three antibodies will help in defining a higher percentage of neoplastic lesions and in evaluating the neoplastic potential of colonic adenomas. In this context it is important to remember that this result was observed when the three antibodies were tested on serial sections from a limited number of carcinomas from different organs [17].

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