Antigen Expressed Alternatively to α-Fetoprotein of Rat Hepatomas

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Rat hepatoma cell line McA Rh 7777 produces α-fetoprotein (AFP) *in vitro*. This line is subcloned into ATP-producing and nonproducing subclones. The AFP-negative clone is used to generate hybridomas producing monoclonal antibodies recognizing AFT$^-$ and AFP$^+$ clones. One of these hybridomas (A2/3) reacts only with AFP$^-$ clones. Double staining for AFP and A2/3 antigen (AG A2/3) reveals alternative expression of AFP and AG A2/3 on McA Rh 7777 hepatoma and its clones. The antigen is present on transplantable H-27 hepatoma (AFP$^-$) and is not expressed by Zaidel hepatoma cells (AFP$^+$). AG A2/3 is absent from the liver of normal adult rats and rat embryos. The antigen is induced *in vivo* by the injection of lead nitrate and *in vitro* by treatment of McA RH 7777 hepatoma and its clones with cadmium chloride. It is likely that AG A2/3 is a cell stress protein.

Key Words: *α-fetoprotein; hepatoma; antigen expressed alternatively to α-fetoprotein*

Numerous tumors synthesize antigens typical of the corresponding normal tissues [1]. The cause of the reexpression of embryonic antigens by tumor cells has not been established. Alpha fetoprotein (AFP) is a typical embryonal glycoprotein expressed by tumors. It is synthesized by the yolk sac entoderm and embryonal liver. This protein is also produced by teratocarcinomas and hepatocellular carcinomas, tumors which are homologous to these tissues [1].

Although regulation of the AFP gene at the cellular and molecular levels have been studied in sufficient detail, the reasons for its reexpression during hepatocarcinogenesis and for considerable differences in its expression by various tumors are unknown [2].

Previously, we managed to clone McA RH 7777 rat hepatoma cell line into clones producing and nonproducing AFP (AFP$^+$ and AFP$^-$, respectively) [3,10]. These clones were used for preparation of monoclonal antibodies (MAB) recognizing AFP$^+$ and AFP$^-$ cells. One of these antibodies (A2/3) reacted with an antigen present in AFP$^-$ cells and absent from AFP$^+$ cells. In the present study it was shown that A2/3 is an inducible hepatic antigen belonging to cell stress proteins.

MATERIALS AND METHODS

Rat hepatoma cell line McA RH 7777 was established by J. Becker *et al.* [8] and kindly donated to the Institute of Carcinogenesis in 1979. These cells actively produce AFP and other serum proteins. They were stored in liquid nitrogen and cultured in Eagle's medium supplemented with 10% fetal calf serum. Experiments were performed on two AFP$^-$ and three AFP$^+$ clones. Two transplantable rat hepatomas were also used: Zaidel ascitic hepatoma (AFP$^+$) and H-27 (AFP$^-$) [7]. Internal organs and embryos at various stages of gestation were collected from outbred rats.

BALB/c mice were immunized with AFP$^-$ hepatoma cells immobilized on a cellulose sorbent [6]. Before immunization, the cells were washed from serum and injected intraperitoneally in a dose of 10^6-

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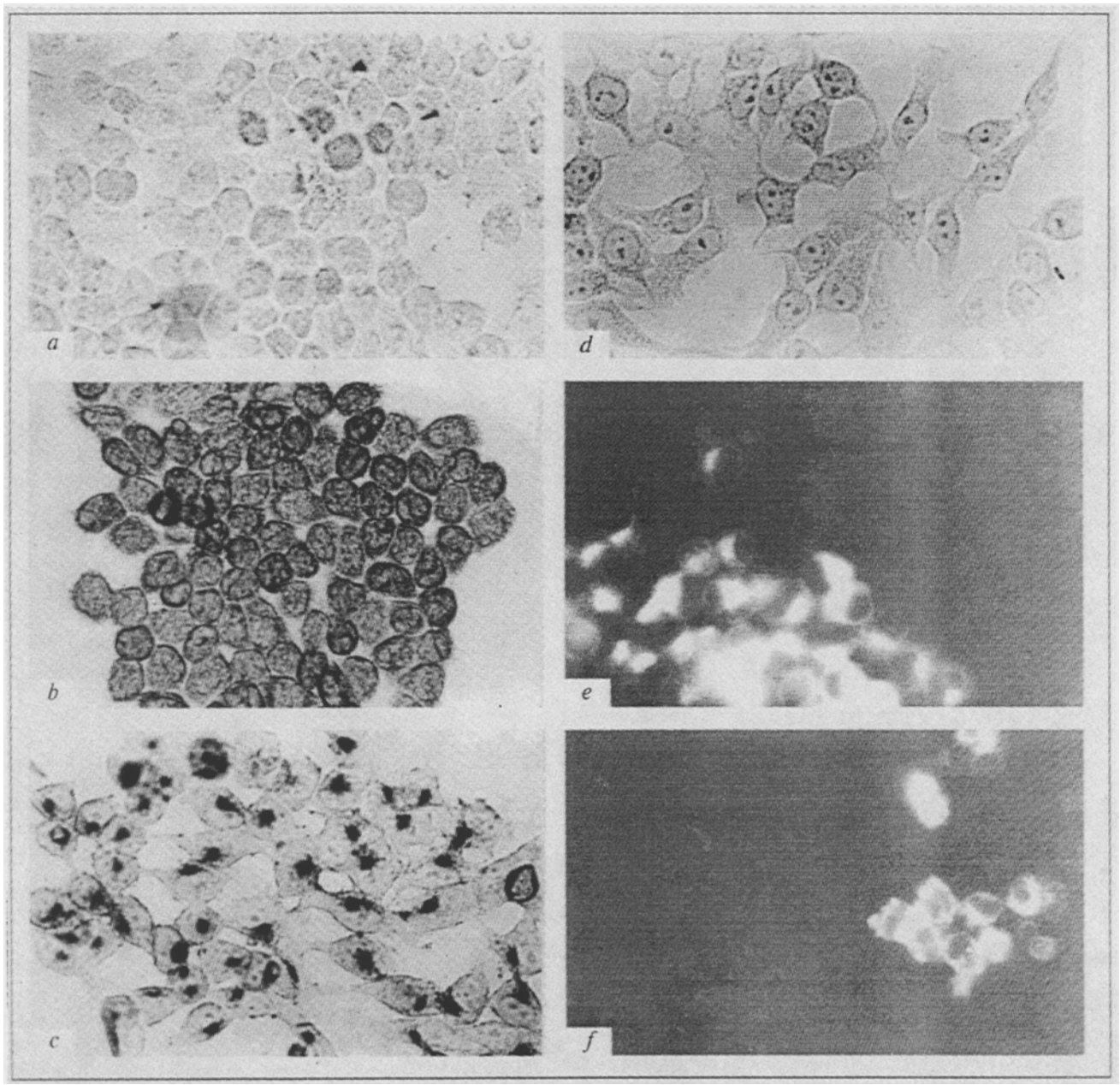

Fig. 1. Localization of α-fetoprotein (*a*, *c*, *e*) and antigen recognized by A2/3 monoclonal antibody (*b*, *d*, *f*) in McA RH 7777 hepatoma clone not producing (*a*, *b*) and producing (*c*, *d*) α-fetoprotein. *e*, *f*) double staining of the same hepatoma cells. ×250.

10^7 cells per animal. The mice were reimmunized after 1 month. Fusion of immune splenocytes with X63Ag8.653 murine myeloma cells was performed as described [9]. Hybridomas were screened by the immunoperoxidase method on AFP^- and AFP^+ cell monolayers. Hybridomas producing MAB discriminating between AFP^- and AFP^+ cells were selected for recloning.

For immunohistochemical analysis hepatoma cells were fixed with 4% paraformaldehyde for 20 min at room temperature and postfixed with 96° ethanol for 15 min. Tissue sections were cut in a cryostat. Cells and tissue sections were fixed with acetone (10 min at 4°C) or 4% paraformaldehyde and incubated first with hybridoma-conditioned culture medium and then with peroxidase-conjugated rabbit anti-mouse Ig (DAKO). Peroxidase activity was revealed by the standard method using 3,3'-diaminobenzidine tetrachloride (Sigma) as a substrate. Rabbit antiserum to rat AFP [3], murine MAB A2/3, and goat antisera to rabbit IgG (TRITC-conjugated, Sigma) and to mouse Ig (FITC-conjugated, Sigma) were used for double immunofluorescence analysis.

In order to asses the *in vitro* effect of Cd^{2+} on McA RH 7777 hepatoma clones, McA RH 7777

monolayers were cultured in the presence of 0.004 mM cadmium chloride. The *in vivo* effects of sovol (chlorinated biphenyl), carbon tetrachloride, and lead nitrate were studied on rats. Sovol in vegetable oil was injected intraperitoneally in a dose of 500 mg/mg. The animals were sacrificed after 4 days [4]. A 20% carbon tetrachloride solution in vegetable oil was injected intraperitoneally in a dose of 0.5 ml/100 g body weight 2 and 4 days before sacrifice. An aqueous solution of lead nitrate was injected into the tail vein in a dose of 10 μmol/100 g body weight. Internal organs were collected after 4 days [11]. Monoclonal antibodies to cytochrome P-450 were prepared by Dr. A. Yu. Kolyada [5].

RESULTS

After screening the hybridomas obtained by fusion of splenocytes of mice immunized with AFP^- McA RH 7777 hepatoma cells with murine myeloma, we selected one hybridoma interesting for the characterization of AFP^- and AFP^+ cells. This hybridoma (A2/3) produced antibodies reacting with AFP^- cells (Fig. 1, *a*, *b*) and not reacting with AFP^+ cells (Fig 1, *c*, *d*). Double staining for AFP and A2/3 antigen (AG A2/3) in McA RH 7777 hepatoma cell line including both AFP^- and AFP^+ cells revealed an alternative distribution of these antigens (Fig 1, *e*, *f*). Thus, the antigen recognized by MAB A2/3 (AG A2/3) can be regarded as a marker of AFP^- cells of McA RH 7777 cell line and its clones. Similar results were obtained in experiments with *in vivo* propagated rat hepatomas: AG A2/3 was not expressed by Zaidel hepatoma cells (AFP^+) and expressed almost by all cells of H-27 hepatoma (AFP^-) (Fig. 2, *a*, *b*).

The antigen A2/3 was not identified in the liver of adult rats, rat embryos, and neonatal rats (Fig. 3, *a*). It was revealed in gastric and intestinal mucosa of some adult rats (Fig. 2, *c*, *d*). The presence of AG A2/3 on some rat hepatomas and its absence from embryonal and adult rat liver suggest that the expression of this antigen can be induced by various stimuli.

In some rats, sovol, an activator of most isoforms of P-450 cytochrome [4], did not induce the expression of AG A2/3 but activated P-450 cytochromes. In other animals, it induced a weak expression of the antigen in some hepatocytes located at blood vessels.

Occasional hepatocytes expressing AG A2/3 were identified in carbon tetrachloride-treated rats. These cells were located in the zone surrounding pericentral

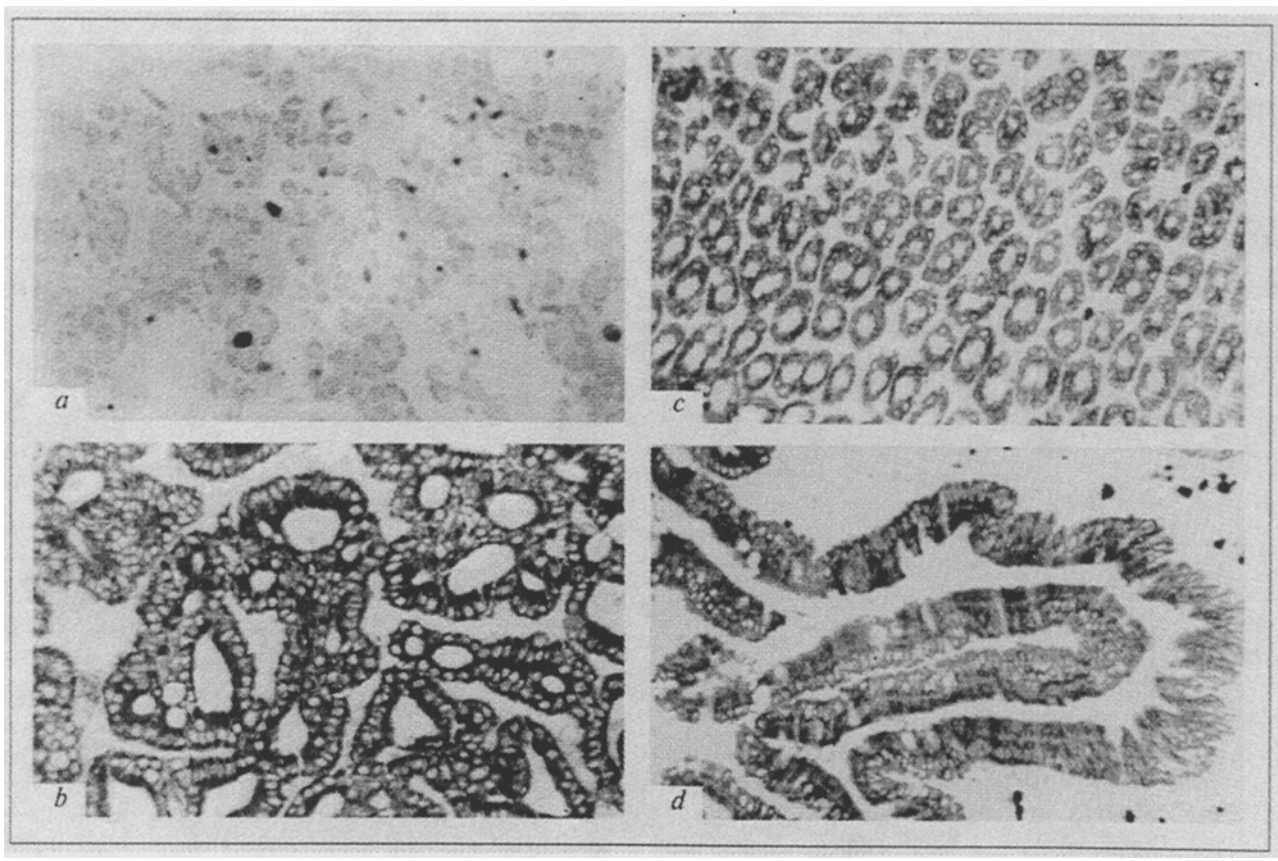

Fig. 2. Localization of the antigen recognized by A2/3 monoclonal antibody on Zaidel (*a*) and H-27 hepatoma cells (*b*), stomach (*c*), and intestine (*d*) of adult rat. ×150.

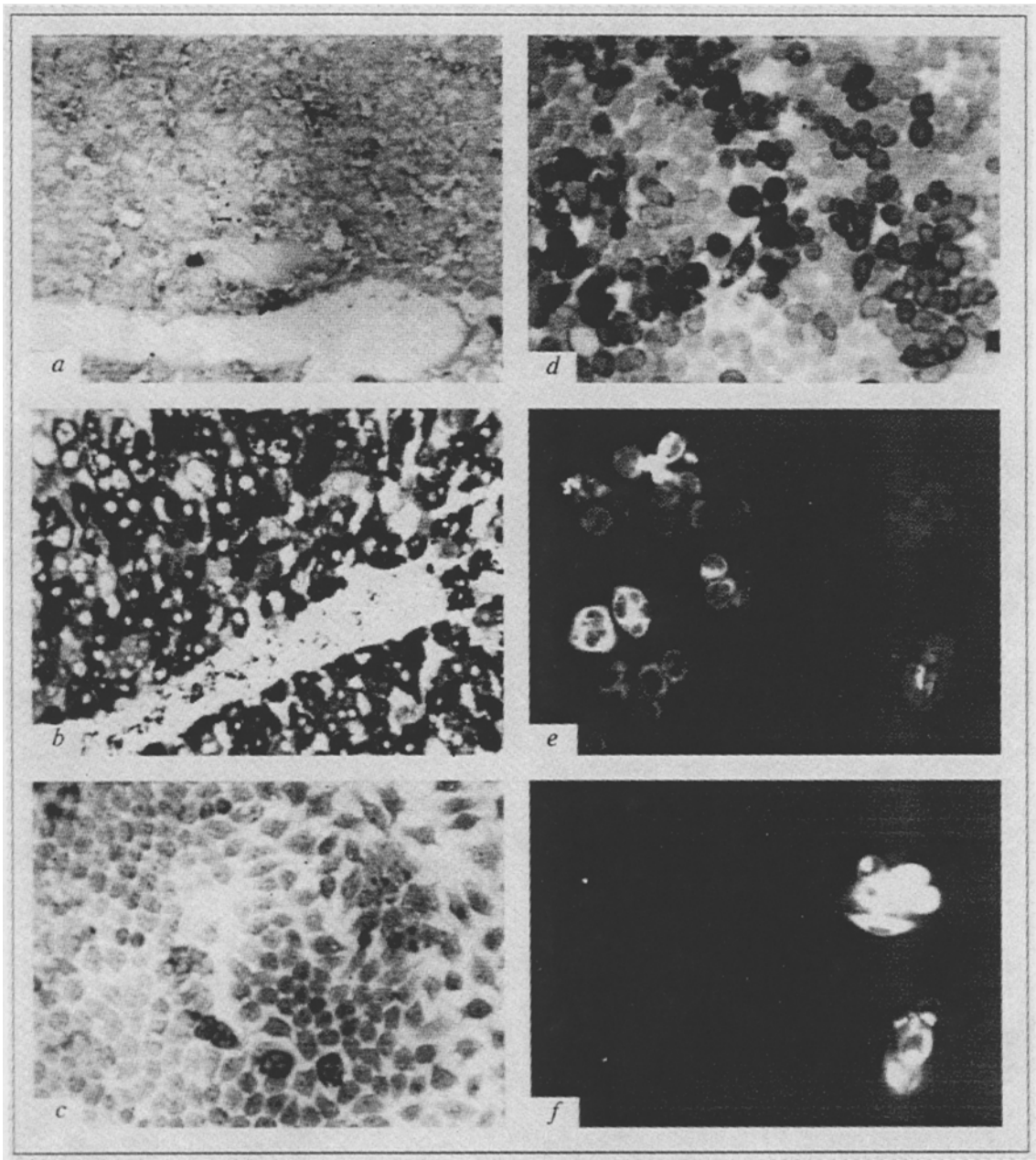

Fig. 3. Induction of A2/3 antigen (AG) *in vivo* and *in vitro*. Localization of AG A2/3 in rat liver before (*a*) and after (*b*) administration of lead nitrate. Localization of AG A2/3 (*c-e*) and α-fetoprotein (*f*) in AG A2/3 negative hepatoma cells before (*c*) and after (*d-f*) culturing in the presence of cadmium chloride. *e*, *f*) double staining for AG A2/3 and α-fetoprotein in the same cells. ×150.

necrosis. Lead nitrate had a pronounced stimulating effect on the expression of AG A2/3 by rat hepatocytes (up to 90%). The intensity of specific staining decreased in the periportal area (Fig. 3, *b*).

The expression of this antigen *in vitro* by AFP$^-$ clones was induced by cadmium chloride (Fig. 3, *c*, *d*). After incubation in the presence of Cd^{2+}, the content of A2/3-positive cells in McA RH 7777 hepatoma cell line increased in parallel with a decrease in the number of AFP-positive cells. The alternative distribution of AFP and AG A2/3 was preserved at the cell level (Fig. 3, *e*, *f*).

These findings indicate that AG A2/3 is an inducible antigen absent from the normal liver and expressed under conditions of cell damage. We think that this antigen does not belong to the cytochrome P-450 family. Presumably, it is a metallothionein, glutathione transferase, or heat shock protein. Anyway, AG A2/3 belongs to cell shock proteins.

Since the epitope reacting with A2/3 MAB is sensitive to SDS, we failed to determine the molecular weight of this protein. However, AG A2/3 can be purified with the use of monoclonal antibodies and sequenced. The cause of the alternative ex-

pression of AFP and AG A2/3 will be established in further investigations.

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Effects of Zoosocial Conflict on Immunological Reactivity of C57Bl/6 Mice

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In aggressive C57Bl/6 mice, the immune response is shown to be enhanced after 20 confrontations with submissive mice. In submissive mice, the response is inhibited after 10-20 confrontations with aggressive partners. It is concluded that stimulation and inhibition of the immune response are associated with the formation of a neurochemical set which is dopaminergic in aggressive mice and serotoninergic in submissive ones.

Key Words: *zoosocial conflict; aggressiveness; submissiveness; immune response*

There is evidence that experimental stressful situations affect immune reactions [4,6]. Changes in the immunoreactivity of animals manifesting natural forms of behavior such as aggressiveness and submissiveness as a result of zoosocial conflicts have not been investigated in sufficient detail. During aggressive confrontations associated with protection of territory, the titers of IgE antibodies in submissive rats are lowered in response to a protein antigen [10], while in submissive C57Bl/6 and DBA mice the production of IgM antibodies is suppressed [12]. It was demonstrated that "high social rank" rats are resistant to infection [11]. Using a model of sensory contact, we showed that the experience of a 10-day-long confrontation stimulates the immune response in aggressive but not in submissive CBA mice in comparison with that in mice which did not participate in conflicts. The immune response was inhibited in submissive C57Bl/6 males after 10 defeats and was not stimulated in aggressive males after 10 victories [1]. With the use of various models it was shown that aggressive behavior is accompanied by activation of

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