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Curative effects on rat sarcomas obtained after a treatment combining two monoclonal antibodies

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The effects of a treatment involving two monoclonal antibodies (Ab) were evaluated on benzo(*a*)pyrene [B(*a*)P]-induced malignant sarcomas in Sprague-Dawley female rats. These Ab were, respectively, an anti-anti-conjugated B(*a*)P AB, an internal image of conjugated B(*a*)P, called AIB1, and an anti-conjugated L-DOPA Ab. They were biweekly injected into animals with small clinically palpable tumors. Anti-'phosphatidylinositol-like' autoantibody (autoAb) levels which were significantly higher in B(*a*)P-treated rat sera were decreased after Ab treatment. Tumor growth was slowed down compared with that of controls and animal survival was increased. This treatment was more efficient than that involving AIB1 alone. The anti-conjugated L-DOPA Ab may play a role in neovascularization, which is known to be critical for tumor growth.

Key words: Benzo(*a*)pyrene, internal image, L-DOPA, monoclonal anti-idiotypic antibody, neovascularization, sarcoma.

Introduction

Increased levels of autoantibody (autoAb) have been found in sera of patients with malignant tumors.[1–4] These autoAb were directed against an endogenous 'benzo(*a*)pyrene [B(*a*)P]-like' structure[3] and a 'phosphatidylinositol (PtdIns)-like' structure.[1,2,4] They reflected disturbances between the endogenous 'B(*a*)P-like' ligand and its cytosolic receptor(s), and disturbances in PtdIns turnover. Moreover, circulating anti-'PtdIns-like' autoAb have been found in sera of Sprague-Dawley (SD) female rats with experimental sarcomas induced by injection of 2 mg B(*a*)P.[5] When a monoclonal anti-idiotypic Ab, an internal image of conjugated B(*a*)P, called AIB1 Ab,[6] was injected into SD female rats, significant anti-'PtdIns-like' autoAb levels appeared. These were found immunochemically equivalent to those found after injection of 2 mg B(*a*)P.[7] Furthermore, AIB1 Ab effects on rat sarcomas have been evaluated before and after the appearance of clinically palpable tumors.[8] The results obtained with this treatment confirmed the relationships between 'B(*a*)P-like' ligand–receptor interactions and PtdIns turnover, and revealed the capacity of a monoclonal anti-idiotypic Ab, an internal image of a ligand involved in tumoral processes, to slow down tumor onset and growth and to increase animal survival.[8]

The present investigation was designed to increase the therapeutic effects of this treatment by simultaneous administration of AIB1 Ab with another monoclonal Ab, the anti-conjugated L-DOPA Ab, which was previously demonstrated to play a role on experimental venous thrombosis.[9] This antibody recognized possible targets located in venous nerve endings, involving modification of the innervation responsible for changes in thrombus formation.[9] We hypothesize that these targets might be involved in various mechanisms, such as angiogenesis, a critical step of tumor growth.[10–12] To support this hypothesis, we have studied the effects of simultaneous administration of AIB1 Ab and anti-conjugated L-DOPA Ab on rat sarcomas. Their ability to modify autoAb levels, to inhibit tumor growth and to increase the life span of treated rats was evaluated and is reported here.

Material and methods

Monoclonal Ab

The three monoclonal Ab used in this study were IgG1 (κ chain). AIB1 Ab, a monoclonal anti-anti-conjugated B(*a*)P Ab, has previously been characterized as the internal image of conjugated B(*a*)P.[6] The monoclonal anti-conjugated L-DOPA Ab and the monoclonal anti-conjugated phencyclidine Ab were obtained as previously described by Chagnaud *et al.*[13] Producing hybridomas were propagated in the peritoneal cavity of BALB/c mice after prior injection with Freund's incomplete adjuvant

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(Difco Laboratories, Detroit, MI; 0.5 ml/mouse). Mouse ascitic fluids were harvested 8–10 days later and mono-clonal Ab partially purified by a 50% $(NH_4)_2SO_4$ precipitation followed by gel filtration (Sephadex G-200).

Sarcoma model

Eighteen female SD rats (Janvier, Le Genest-Saint-Isle, France) were housed under controlled conditions (22°C, monitored light–dark cycles, with light on from 8:00 a.m. to 8:00 p.m.) and were supplied with food (UAR, Versailles, France) and water *ad libitum*. The 50–60 day old female SD rats weighing 180–200 g received under anesthesia a single s.c. injection of 2 mg of B(*a*)P, diluted in 500 µl of sesame oil, at the top of the right thigh.[5] Blood of B(*a*)P-treated rats was regularly sampled and tested (Figure 1).

Ab treatment

Ab solutions were prepared as follows: G-200 purified fractions of Ab were diluted at 2 mg/ml in NaCl 9‰ (Meram SA, Melun, France) and stored at −80°C. At day 115 after B(*a*)P administration, all SD female rats had small clinically palpable tumors (volume around 65 mm^3). They were divided in three groups and were biweekly s.c. injected at the top of the left thigh with either 500 µg of AIB1 Ab plus 500 µg of anti-conjugated L-DOPA Ab ($n=7$, group 1), 500 µg of AIB1 Ab alone ($n=4$, group 2) or 500 µg of anti-conjugated phencyclidine Ab ($n=7$, control group). Treatment was continued until the death of each animal (Figure 1).

Immunoenzymatic method

Detection of anti-'PtdIns-like' autoAb was performed with an adapted ELISA method as previously described by Faiderbe *et al.*[5] on rat sera diluted at 1/1000. Experimental absorbance values obtained from well-plates coated with PtdIns were corrected by subtracting blank values read from wells coated with control protein (thyroglobulin).

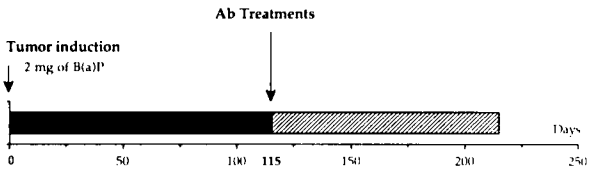

Figure 1. Description of the experimental protocol. Time sequence of the events (tumor induction, time course of anti-'PtdIns-like' autoAb, tumor appearance) and Ab treatments. ▨, time course of autoAb; ■, tumor appearance; ▨, biweekly administration of Ab.

Histological analysis

Some animals were randomly chosen in each group and their tumors were removed, cut in pieces and fixed in Bouin de Hollande.

Tumor growth measurement

B(*a*)P-induced tumors were mechanically measured with a calliper rule as described by Faiderbe *et al.*[5] Their volume was calculated using a standard formula: width^2 × length × 0.52, according to Ingber *et al.*,[10] and expressed as cubic millimeters.

Results

Effects of Ab treatment on circulating anti-'PtdIns-like' autoAb levels

Detection of anti-'PtdIns-like' autoAb levels was performed as previously described in Material and methods in order to monitor the malignant transformation and effects of AIB1 Ab plus anti-conjugated L-DOPA Ab treatment (Figure 2). B(*a*)P-treated rat anti-'PtdIns-like' autoAb levels greatly increased 40 days after B(*a*)P administration. AutoAb levels increased until day 100 and then plateaued (Figure 2, curve 3). When AIB1 Ab plus anti-conjugated L-DOPA Ab solution was administered 115 days after carcinogen injection [optimal autoAb titer, small clinically palpable tumors (around 65 mm^3)], anti-'PtdIns-like' autoAb levels decreased around 70% (Figure 2, curve 1) in relation to the control group treated with anti-conjugated phencyclidine Ab (Figure 2, curve 3). Anti-'PtdIns-like' autoAb levels remained at a low level (between 70 and 50% of the control level) until day 175 corresponding to a treatment of 16 injections of Ab. From that day onwards, although Ab injections were carried on, the autoAb levels of treated rats reached those of control rats. For rats treated with AIB1 Ab alone, anti-'PtdIns-like' autoAb levels decreased only 30% (Figure 2, curve 2), thereby confirming our previous results.[8]

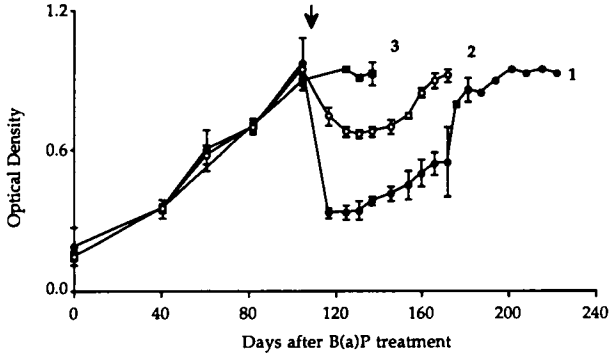

Figure 2. Effects of AIB1 Ab plus anti-conjugated L-DOPA Ab treatment on circulating anti-'PtdIns-like' autoAb levels in rat sera. Optical density represents the binding of rat autoAb (1/1000) to PtdIns coated on well plates. Curves show mean optical density values with standard error. Curve 1 (●), AIB1 Ab plus anti-conjugated L-DOPA Ab treatment 115 days after B(*a*)P administration ($n = 7$); curve 2 (○), AIB1 Ab treatment 115 days after B(*a*)P administration ($n = 4$); curve 3 (■), anti-conjugated phencyclidine Ab treatment 115 days after B(*a*)P administration (control group, $n = 7$). →: beginning of Ab treatment.

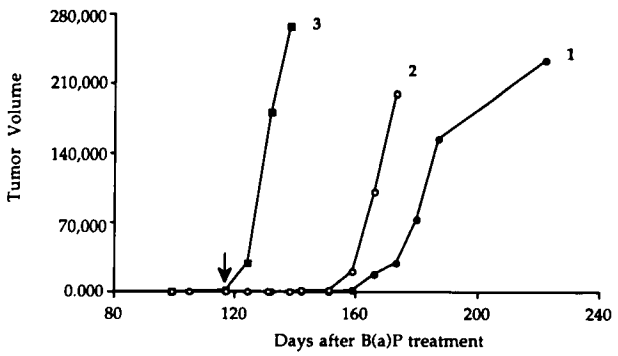

Figure 3. Time course of tumor growth in AIB1 Ab plus anti-conjugated L-DOPA Ab treatment 115 days after B(*a*)P administration (curve 1, ●), AIB1 Ab treatment 115 days after B(*a*)P administration (curve 2, ○) and anti-conjugated phencyclidine Ab treatment 115 days after B(*a*)P administration (curve 3, ■). The tumor volume was calculated with the formula: width^2 × length × 0.52, and is expressed as mm^3. →: beginning of Ab treatment.

Effects of Ab treatment on tumor growth evolution

At day 115, all SD female rats had small clinically palpable tumors (approximately 65 mm^3) that were regularly examined. Tumors were mechanically measured as previously described in Material and methods. Figure 3 shows the increase of tumor growth for the three groups treated with AIB1 Ab plus anti-conjugated L-DOPA Ab, AIB1 alone or anti-conjugated phencyclidine Ab (control group). Ten days after detection of clinically palpable tumors, control rat tumor volumes rapidly increased (Figure 3, curve 3). The animals did not survive beyond day 25 after the beginning of the experiment. For rats with small clinically palpable tumors treated with AIB1 Ab plus anti-conjugated L-DOPA Ab from day 115, tumor volumes increased until day 175, more slowly than those of the control group (Figure 3, curve 1). For rats with small clinically palpable tumors at day 115 treated with AIB1 alone, tumor volumes increased until day 159, more slowly than those in the control group (Figure 3, curve 2). The simultaneous administration of AIB1 Ab and anti-conjugated L-DOPA Ab had a better decelerating effect on tumor growth (Figure 3, curves 1 and 2).

Effects of Ab treatment on animal survival

Survival curves were drawn with the non-parametric Kaplan–Meier test from these data. Figure 4 shows that the mean survival of the AIB1 Ab plus anti-conjugated L-DOPA Ab treated rats (curve 1) was increased by about 80 days relative to the control group (curve 3). Moreover, statistical analysis performed with the log–rank test (Mantel Cox) revealed that the mean survival of AIB1 Ab plus anti-conjugated L-DOPA Ab treated rats was significantly increased ($p = 0.002$). For rats treated with AIB1 alone, the mean survival was significantly increased ($p = 0.002$) by about 30 days relative to the control group (Figure 4, curve 2), as previously observed.[8]

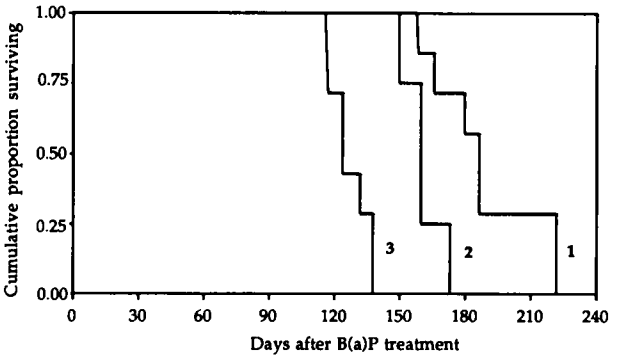

Figure 4. Effects of AIB1 Ab plus anti-conjugated L-DOPA Ab treatment on animal survival. Cumulative proportion surviving in the AIB1 Ab plus anti-conjugated L-DOPA Ab treated rat group, AIB1 Ab alone treated rat group and the control group with clinically palpable tumors at the beginning of treatment, expressed as percentage of surviving animals during the time course of experimentation. (1) AIB1 Ab plus anti-conjugated L-DOPA Ab treated rats, (2) AIB1 Ab alone treated rats and (3) control group.

Histological analysis

Histological analysis of the tumors showed the presence of highly malignant sarcomas with fusiform cells for control rats, as previously described by Faiderbe *et al.*,[5] and sarcomas with cells presenting no morphological changes whatever the Ab treatment used: AIB1 Ab plus anti-conjugated L-DOPA Ab or AIB1 Ab alone.

Discussion

With our tumor model induced by a single dose of 2 mg of B(*a*)P, an increase of anti-'PtdIns-like' autoAb titers was seen in all B(*a*)P-treated SD female rats. All the animals developed a highly malignant sarcoma at the carcinogen injection site (top of the right thigh) around 100–120 days after B(*a*)P administration.[5] Tumor incidence and/or growth were easily monitored prior to death. This model was thus reliable for the evaluation of Ab treatment effects (Figure 1).

In previous work, we demonstrated that AIB1 Ab, a monoclonal anti-idiotypic Ab, an internal image of conjugated B(*a*)P,[6] has the capacity to delay tumor onset, slow down growth and to increase animal survival when administered biweekly to SD female rats, at different times after B(*a*)P administration.[8]

To increase the curative effects observed with AIB1 Ab treatment, simultaneous administration of anti-conjugated L-DOPA Ab was assayed. As previously demonstrated by Lagier *et al.*,[9] the *in vivo* administration of anti-conjugated L-DOPA Ab modifies venous thrombosis formation in a rat model. The authors proposed the possible existence of endogenous compounds with conjugated 'L-DOPA-like' epitopes located in nerve endings of veins. The recognition of these possible targets by the monoclonal anti-conjugated L-DOPA Ab could involve various mechanisms like modification of the venous innervation and the authors reported that the observed effects on thrombus formation might be the result of a variation in venomotricity. These possible targets might also be involved in new blood vessel genesis, which is particularly required in the rapid growth of solid tumors. Some authors have previously established that unrestricted growth of tumors depends upon angiogenesis.[10–12] Kim *et al.* reported that treatment with a monoclonal Ab specific for the vascular endothelial growth factor supposed to be an angiogenic factor may suppress tumor growth *in vivo*.[12] Ingber *et al.*[10] showed that synthetic analogues of fumagillin, a naturally secreted antibiotic of *Aspergillus fumigatus* fresenius, inhibits tumor-induced angiogenesis in the subcutaneous dorsal air sac of the mouse.

When AIB1 Ab plus anti-conjugated L-DOPA Ab solution was administered biweekly to SD female rats with small clinically palpable tumors (65 mm^3), anti-'PtdIns-like' autoAb levels decreased around 70% in relation to the control group (Figure 2, curve 1). This decrease was greater than that observed in SD female rats with small clinically palpable tumors (65 mm^3) treated with AIB1 Ab alone.[8] In this case the anti-'PtdIns-like' autoAb levels decreased only by 30% (Figure 2, curve 2). A comparative decrease in anti-'PtdIns-like' autoAb level (around 80%) was obtained in SD female rats treated with AIB1 Ab alone, when this latter was biweekly administered 73 days after B(*a*)P injection (optimal autoAb titer, no tumor).[8]

For AIB1 Ab plus anti-conjugated L-DOPA Ab treated rats with small clinically palpable tumors (65 mm^3) at day 115, tumor growth slowed down in relation to that of controls (Figure 3, curve 1). Animal survival was significantly increased by about 80 days relative to the control group (Figure 4, curve 1). For SD female rats with small clinically palpable tumors (65 mm^3) treated with AIB1 Ab alone, tumor volume increased more slowly than in the control group and animal survival was significantly increased by only 32 days relative to controls (Figure 4, curve 2), thus confirming our previous data.[8] For AIB1 Ab plus anti-conjugated L-DOPA Ab treatment, anti-'PtdIns-like' autoAb remained at a low level until day 175. From that day to animal death, they reached those of controls (Figure 2, curve 1). Similarly, from day 175 to death, tumors drastically increased to reach sizes equivalent to those of controls (Figure 3, curve 1). The comparison between tumor growth and anti-'PtdIns-like' autoAb levels, over this period of time, strongly confirmed the value of such a criterion (anti-'PtdIns-like' autoAb levels) to monitor malignant transformation.

AIB1 Ab plus anti-conjugated L-DOPA Ab administration showed no major toxicity whatever the treatment schedule used. A possible toxicity of AIB1 Ab treatment has previously been envisaged, since some animals treated from day 73 with AIB1 Ab alone died with small tumors, around 100 days after the beginning of the treatment.[8] Under the experimental conditions described here, rats were treated with AIB1 Ab plus anti-conjugated L-DOPA Ab during an equivalent period (around 105 days) and they died with tumors equivalent in size to those of controls. the possible toxicity of AIB1 seems to be unlikely.

Our results show that effectiveness of the curative treatment with AIB1 Ab on SD female rats was increased by simultaneous administration with an anti-conjugated L-DOPA Ab. The administration of anti-conjugated L-DOPA Ab alone had no influence on anti-'PtdIns-like' autoAb levels and tumor growth evolution, under our experimental conditions (data not shown). It is unclear whether anti-conjugated L-DOPA Ab actually mediates angiogenesis and tumor growth *in vivo*. The inability of solid tumors to grow to a clinically significant size in the absence of the successful induction of angiogenesis has prompted a long-standing debate regarding the induction of compounds that block neovascularization. Anti-inflammatory agents,[14] protamine,[15] angiostatic steroids,[16] and a variety of compounds that influence matrix synthesis and integrity[17,18] have been identified as inhibitors of neovascularization. Several of these inhibitors have been shown to limit tumor growth or to induce tumor regression *in vivo*,[15,17] although not all tumors respond and toxicity restricted their use. Proof of the role of L-DOPA-like targets in tumor angiogenesis requires the demonstration that their inhibition prevents tumor growth *in vivo*. The availability of the anti-conjugated L-DOPA Ab capable of blocking 'L-DOPA-like' target-induced angiogenesis *in vivo* and *in vitro* will allow us to test this hypothesis directly.

Folkman *et al.* have demonstrated that the induction of angiogenesis and consequently neovascularization both precede tumor formation, and that they correlate with the transition from hyperplasia to neoplasia.[11] So it will be interesting to test on our sarcoma model the effects of AIB1 Ab plus anti-conjugated L-DOPA Ab treatment at different times after B(*a*)P administration. Beginning the treatment 70 days after B(*a*)P administration (optimal autoAb levels and no tumor) should be revealing. At that moment, the best response regarding onset and growth is obtained with AIB1 alone.[8] Furthermore at this stage of tumor development, it could be that angiogenesis is not induced and that anti-conjugated L-DOPA Ab is more effective.

Conclusion

Our results confirm the relationships between metabolic pathways involving possible 'B(*a*)P-like' endogenous ligand-cytosolic receptor(s) and the PtdIns. AIB1 Ab effects revealed the capacity of a monoclonal anti-idiotypic Ab, an internal image of a ligand involved in tumoral processes, to slow down tumor growth and to increase animal survival. Furthermore, these results show that curative treatment with AIB1 Ab is more effective when an anti-conjugated L-DOPA Ab is administered simultaneously. Although the possible anti-angiogenesis role of an anti-conjugated L-DOPA Ab is suggested here but not demonstrated, our findings provide a glimpse of what a cancer therapy based on both anti-angiogenesis and anti-tumoral growth might be in the future.

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