Identification and Characterization of a Specific Autoantiphosphatidylinositol Immune Response during the Time Course of Benzo(a)pyrene-induced Malignant Tumors in Female Sprague-Dawley Rats

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ABSTRACT

High levels of anti-phosphatidylinositol (PtdIns) autoantibodies (autoAb) have been previously described in sera of cancer patients and in plasma of dimethylbenzanthracene-treated female Sprague-Dawley rats. The presence of anti-PtdIns autoAb was tested in a model of highly malignant sarcomas induced by a single dose of benzo(a)pyrene [B(a)P] diluted in sesame oil and injected in female Sprague-Dawley rats. Significantly elevated levels of anti-PtdIns autoAb were found in sera of B(a)P-treated rats 40 days after B(a)P administration, whereas no significant levels of anti-PtdIns autoAb were noted in oil- or benzo(e)pyrene-treated rats. After day 60, autoantibody levels plateaued in B(a)P-treated rats, and highly malignant sarcomas appeared with 100% efficiency around day 100. Anti-PtdIns autoAb avidity and specificity were found to be high.

INTRODUCTION

Circulating autoAb and immune complexes have been found in sera of patients with malignant tumors (1–7) and animals with experimental tumors (8, 9). However, most results have been hampered by the lack of characterization of Ag recognized by the Ab. Recently, we have found the presence of circulating autoAbs directed against a chemically defined Ag, PtdIns, in highly diluted human sera (1/15,000) of patients with malignant tumors (10, 11) and in plasma (1/1000) of female SD rats with DMBA-induced mammary tumors (12). Human and rat anti-PtdIns autoAb recognition was evaluated by competition experiments with ELISA, and autoAb were found to have identical specificity, suggesting that the same immune processes may be induced during cell proliferation and malignant transformation. Moreover, the early increase of anti-PtdIns autoAb in DMBA-treated rat plasma points to their predictive value (12). To see if anti-PtdIns autoAb may be evidenced in another model of tumors induced in female SD rats treated with another potent carcinogenic PAH, we injected B(a)P s.c. in these rats and monitored the appearance of anti-PtdIns Ab. Anti-PtdIns autoAb predictive value was evaluated. To determine whether the anti-PtdIns autoAb increase is due to malignant cell transformation or to the administration of a PAH molecule, we treated female SD rats with a noncarcinogenic PAH, B(e)P, and compared the in vivo effects of the two PAHs.

MATERIALS AND METHODS

Chemicals. B(a)P, B(e)P, PtdIns, and PtdIns-related compounds Ins, PtdIns4P, and PtdIns4,5P2 were purchased from Sigma Chemical Co. (St Louis, MO), as were other PL and proteins (TH, bovine serum albumin, and human serum albumin). Rabbit anti-rat Ab labeled with horseradish peroxidase were obtained from Miles.

Animal Injection and Sample Collection. Twenty-four female SD rats, divided into three groups [B(a)P-, B(e)P-, and oil-treated groups] (Iffa-Credo, Lyon, France), were housed under controlled conditions (22°C; monitored light-dark cycles, with lights on from 7:00 a.m. to 7:00 p.m.). The 50–60-day-old female SD rats, weighing 180–200 g, were supplied with food (UAR, Versailles, France) and water ad libitum.

Two mg B(a)P or B(e)P, diluted in 500 μl of sesame oil, or 500 μl of carrier solution (oil) were administered to SD rats under anesthesia, by a single s.c. injection at the top of the right thigh. Blood of treated rats was regularly sampled and tested.

Immunoenzymatic Test for Detection and Characterization of Anti-PtdIns AutoAb in Rat Serum. The detection of anti-PtdIns autoAb was performed with an adapted ELISA. The amphotic nature of the PtdIns molecules and their rather high molecular weight (M, > 1000) made their use possible without conjugation. Different compounds were tested as controls (blank value), in order to optimize the ELISA: TH, bovine serum albumin, human serum albumin, and other PL (phosphatidylethanolamine, phosphatidylycerine, and phosphatidylcholine). The best ratio of specific absorbance to background was obtained when TH coated on wells was used as a blank value. Polystyrene wells (Nunc) were coated with 200 μl of either PtdIns (10 μg/ml) or TH (10 μg/ml) in 10⁻² M phosphate solution with 10⁻³ M CaCl₂, pH 7.0, for 16 h at 37°C. The wells were washed twice with a 10⁻² M phosphate, 0.15 M NaCl buffer (PBS), pH 7.4, and were incubated at 37°C for 2 h with 200 μl of SD rat serum diluted 1000-fold in PBS plus 10% glycerol. The plates were rinsed twice with PBS and incubated for 1 h at 37°C with rabbit immunglobulin anti-rat IgG(γ) labeled with horseradish peroxidase, diluted 5000-fold. Peroxidase was assayed as described by Faiderbe et al. (12). Experimental absorbance values obtained from wells coated with PtdIns were corrected by subtracting blank values of wells coated with TH. Rat anti-PtdIns autoAb avidity and specificity were evaluated by competition experiments between PtdIns coated on wells and PtdIns or other compounds [Ins, PtdIns4P, PtdIns4,5P₂, or other PL (phosphatidylycerine, phosphatidylcholine, or phosphatidylethanolamine)] diluted to 10⁻³ M to 10⁻¹ M and previously preincubated for 16 h at 4°C with rat serum diluted at 1/1000.

Purification of SD Rat Immunglobulins and Determination of Anti-PtdIns Ab Isotype. Sera from B(a)P- or oil-treated SD rats were purified with 50% (NH₄)₂SO₄ precipitation, followed by Sephadex G-200 (Pharmacia) chromatography in 10⁻² M phosphate buffer containing 4 liter/g NaCl. Each fraction was assayed in our ELISA. Rat IgM, IgA, or IgG binding to PtdIns was revealed by addition of rabbit immunglobulin anti-rat IgG(γ), -A(α), or -M(α) labeled with horseradish peroxidase (Miles).

Histological Analysis. After 150 days, tumors were removed, cut in pieces, and fixed in Bouin de Hollande.

Tumor Growth Measurement. B(a)P-induced tumors were measured in two dimensions. Their volume was calculated using a standard formula, width² × length × 0.52, according to the method of Ingber et al. (13).

Statistical Tests. Levels of autoantibodies were given as means of absorbances, with the SE resulting from triplicate determinations.
RESULTS

Evaluation of Anti-PtdIns AutoAb Levels after Treatment.

Every 10 days, groups of eight experimental [B(a)P- or B(e)P-treated] and control (oil-treated) animals were bled. Immunological binding was evaluated in rat sera diluted 1000-fold, on plates coated with PtdIns and TH. Fig. 1 shows that the mean autoAb levels of B(a)P-treated rats (Fig. 1, curve 1) were greater than those of B(e)P- or oil-treated rats, beyond day 10. B(a)P-treated rat autoAb levels appeared statistically significant (α < 0.05, U test) 40 days after B(a)P treatment, and the ratio of the means [defined as mean absorbance value of B(a)P- or B(e)P-treated rats minus mean absorbance value of oil-treated rats/ mean absorbance value of oil-treated rats] was found to be 1. Levels increased until day 60, when they plateaued. The ratio was found to be, respectively, 1.9 and 0.6 for B(a)P- and B(e)P-treated rat sera at day 60. Mean absorbance values of B(e)P-treated rat Ab (Fig. 1, curve 2) remained at the level of those of control rats (Fig. 1, curve 3).

Isotypic Characterization of Rat Anti-PtdIns AutoAb. Sera of B(a)P- or oil-treated rats (n = 2 for each group, bled at day 60) were chromatographed on Sephadex G-200. Each fraction was diluted to 0.5 mg/ml and assayed with our ELISA. Rat anti-PtdIns autoAb binding was revealed by different anti-rat immunoglobulin isotypes labeled with peroxidase (Fig. 2). For B(a)P-treated rat sera, immunological binding was found with fractions corresponding to the IgG elution peak, since immunoglobulin isotype was revealed with rabbit anti-rat IgG(γ) Ab. No immunological binding was seen in the IgA and IgM elution peaks, as revealed with rabbit anti-rat IgA(α) or IgM(μ). At the same immunoglobulin concentration for oil-treated rat sera, the immunological response of the IgG elution peak was 4.8 times less than that of B(a)P-treated rat sera, corresponding to the same ratio found with crude rat sera (see Fig. 1). Isotypic characterization strongly indicates that anti-PtdIns Ab found in B(a)P-treated rat sera are IgG.

Immunochromatography of Anti-PtdIns AutoAb. Anti-PtdIns autoAb avidity and specificity of B(a)P- or B(e)P-treated and control rat sera were evaluated by competition experiments, as described in “Materials and Methods,” using rat sera collected 60 days after treatment, i.e., when the anti-PtdIns autoAb levels plateaued. For this, they were diluted 1000-fold, preincubated or not with different competitors (PtdIns, PtdIns4P, PtdIns4,5P2, Ins, and other PL), and applied to wells coated with PtdIns and TH. For B(a)P-treated rat sera, the displacement curves are plotted in Fig. 3. Avidity was defined as competitor concentration at half-displacement. The best displacement was observed when B(a)P-treated rat serum was preincubated with PtdIns. This occurred at a concentration of 8 × 10^{-10} M (Fig. 3, curve 1). PtdIns-related compounds were less well recognized [22-fold less for PtdIns4,5P2 (Fig. 3, curve 2), 50-fold less for PtdIns4P and Ins (Fig. 3, curves 3 and 4)] than PtdIns itself. The other PL (phosphatidylserine, phosphatidylcholine, and phosphatidylethanolamine) were not recognized at all (cross-reactivity ratios of ~125,000; Fig. 3, curve 5). For B(e)P- or oil-treated rat sera, no displacement was observed whether PtdIns, related compounds (Ins, PtdIns4P, or PtdIns4,5P2), or other PL were used (curves not shown). These latter results are related to the polyspecific character of the B(e)P-treated or control rat immunoglobulins, in comparison to those of B(a)P-treated rats.

Histological Analysis. Around days 100–120, all B(a)P-treated rats developed tumors, with 100% efficiency, at the site...
of injection (top of the right thigh). Each histological analysis showed the presence of a highly malignant sarcoma. B(e)P- and oil-treated rats did not develop any tumor, even a long time after treatment (>200 days).

Comparison of Tumor Growth and Anti-PtdIns AutoAb Levels.

Fig. 4 shows the evolution of anti-PtdIns autoAb levels and tumor growth for a B(a)P-treated SD rat, taken as an example. The Ab levels, which plateaued at day 60, remained stable, while the tumor drastically increased from day 100 to animal death. The same profile of tumor growth evolution was observed for each B(a)P-treated female SD rat.

DISCUSSION

B(a)P has been tested for carcinogenicity many times in several animal species and by various routes of administration (14–16). Well differentiated sarcomas that developed in s.c. tissues of different strains of mice (BALB/c, C3H) following a single injection of B(a)P (0.004 mg/g body weight) were observed, with an incidence varying between 20% and 50% (17). In the present report, a B(a)P-induced malignant tumor model is described. It was obtained after the s.c. administration (top of the right thigh of the animals) of a single dose of B(a)P (0.01 mg/g body weight) diluted in sesame oil. All female SD rats developed a highly malignant sarcoma between 100 and 130 days after B(a)P treatment.

This model of highly malignant sarcoma enabled us to monitor the appearance and increase of anti-PtdIns autoAb levels in sera during the course of cell transformation and proliferation. Our data confirm and extend those previously described (12); B(a)P, like DMBA, caused an increase in circulating anti-PtdIns autoAb in sera of PAH-treated animals, whereas administration of a single dose of sesame oil did not modify the titer of natural polyspecific Ab in female SD rat sera (Fig. 1). Moreover, no correlation was found between tumor size and anti-PtdIns autoAb levels in rat sera (Fig. 4). Additionally, in comparison with other malignant tumor markers, which increase with tumor growth, anti-PtdIns autoAb levels in B(a)P-treated rat sera were significantly higher than those in oil-treated rat sera from 40 days after treatment and plateaued 60 days after carcinogen treatment, whereas the animals did not yet have visible tumors (Fig. 1). Consequently, it is tempting to conclude that autoimmune processes with PtdIns might have a predictive value in B(a)P carcinogenesis.

These autoAb were of the G class (Fig. 2) and identical in specificity (Fig. 3), whatever the carcinogen or tumor type (sarcoma or carcinoma), in rats and humans (10–12). The glyceryl phosphate-inositol part of the neoantigen has always been found to be immunodominant. Such an immunological response could be explained by the constant stimulation of lymphocytes reactive for PtdIns by an endogenous Ag, of which PtdIns could be one of the components. Our results are consistent with this hypothesis. In fact, it is unlikely that the penetration of PAH through the membrane lipids (18, 19) and the consequent local inflammatory processes could, alone, provoke such an increase in anti-PtdIns Ab levels, since no significant autoAb levels were noted in B(e)P-treated rat sera.

Thus, the neosynthesis of PtdIns Ag and its recognition by the lymphocytes might be understood by considering that (a) during cell proliferation, PtdIns4,5P2 hyperproduction has been shown to be enhanced by some types of cellular and viral oncogenes (20), (b) in malignant tumors phosphoinositide metabolite levels have been found to be altered (21), and (c) phospholipase C and D activities, with PtdIns or PtdIns4,5P2 as substrate, are manifold higher in cancer cells (21–24). Any modification in PtdIns metabolism might contribute to the neosynthesis of PtdIns Ag and, consequently, to the appearance of anti-PtdIns autoAb during malignant cell transformation and proliferation.

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