Modulation of DNA Binding in Vivo by Specific Humoral Immunological Response: A Novel Host Factor in Environmental Carcinogenesis?

Alessandra Verdina,1 Romano Zito,1 Giancarlo Cortese,1 Andrea Zijno,2 and Riccardo Crebello2

1Centro Ricerche Sperimentali, Istituto Regina Elena; 2Istituto Superiore di Sanita', Rome, Italy

To investigate the possible modulatory effect of the immune response induced by recurrent carcogen exposure, a specific humoral immune response toward 2-acetylaminofluorene (2-AAF) was elicited in Swiss mice with repeated intraperitoneal injections of a 2-AAF–gelatin conjugate. The immunization procedure resulted in the production of specific anti-2-AAF antibodies in all treated animals. Groups of immunized and nonimmunized mice were subsequently fed 2-AAF pelleted in the diet at 50 and 150 ppm for 4 weeks. At the end of 2-AAF administration, animals were sacrificed and the content of 2-AAF–adducts in liver DNA was determined by enzyme-linked immunoadsorbent assay using a polyclonal rabbit antisera. The comparison of the adduct levels in immunized and nonimmunized mice (receiving either the vehicle or the adjuvant alone during pretreatment) demonstrates a highly significant (p < 0.001) difference among groups, with far lower adduct levels in immunized animals. No significant difference in food consumption or liver metabolic activities was observed among experimental groups, suggesting the absence of external bias. The mechanism underlying the result observed is not yet clear; however, the experimental data strongly suggest that the specific immunological response induced by recurrent carcogen exposure may exert a modulatory effect and act as a relevant host factor in chemical carcinogenesis. — Environ Health Perspect 104(Suppl 3):679–682 (1996)

Key words: humoral immunity, 2-acetylaminofluorene, carcogen–protein conjugate, DNA binding

Introduction

Manifold inherited and acquired host factors such as variance in DNA repair (1–3) and xenobiotic metabolism (4–6), age, nutrition, stress, diseases, hormonal status (7–8), and immunological (9) and genetic factors (10,11) are known to affect the individual response to chemical carcinogens (12–14). Another significant trait might be represented by the immunological response elicited by the formation of adducts to macromolecules during chronic carcinogen exposure. In this regard, previous studies demonstrated the presence of antibodies directed against benzopyrene–DNA adduct in blood sera of humans occupationally exposed to high levels of polycyclic aromatic hydrocarbons (PAHs) (15–16) and in the urban population (17). Consequently, this trait has been proposed as a retrospective individual exposure marker in biomonitoring studies (18). On the other hand, it has not yet been elucidated whether such induced immune response may play some mechanistic role, such as modulating the effect produced by the carcogen itself. An interesting clue to this possibility was previously provided by the observation of a protective effect of the secretory immune response to 2-acetylaminofluorene (2-AAF) in rabbits, possibly related to the reduction in transepithelial absorption of the carcogen (19).

To investigate the potential significance of the humoral anticarcinogen immunity, a specific immunological response toward 2-AAF was elicited in Swiss mice. Both immunized and nonimmunized animals were subsequently challenged with a 4-week dietary exposure to the carcogen. At the end of treatment, 2-AAF binding to liver DNA was determined in all animals and evaluated in relation to their immune status and carcogen exposure.

Methods

Animals

Male Swiss mice (Charles River, Calco, Como, Italy), 4 weeks of age at the beginning of treatment, were maintained on a balanced standard chow (Mucedola, Milan, Italy) and tap water ad libitum. Animal care, treatments, and sacrifice were conducted in strict accordance with Directive 86/609/EEC on the protection of laboratory animals.

Immunization

A 2-AAF-gelatin conjugate (20) was used as the immunogen. The immunization procedure consisted of three weekly intraperitoneal (ip) administrations of the complete immunogen (50 μl of 2-AAF–gelatin conjugate 1 mg/ml together with 50 μl of Freund’s adjuvant). A further injection was delivered 14 days after the third treatment. Controls received the adjuvant alone or no treatment.

2-AAF Treatment

One week after the end of the immunization, both immunized and nonimmunized mice were fed 2-AAF pelleted in the diet at 50 and 150 ppm for 4 weeks. Parallel control groups were fed with the standard diet.

Determination of 2-AAF Adducts to Liver DNA

DNA Extraction. Livers frozen at −80°C were thawed and homogenized in extraction buffer (10 mM Tris–HCl, pH 8.0, 100 mM EDTA, 0.5% sodium dodecyl sulfate [SDS], 20 μg/ml RNase) and incubated 1 hr at 37°C before the addition of protease K (Sigma Chemical Co., St. Louis, MO) to a final concentration of 100 μg/ml. After 2 hr of incubation at 50°C, DNA was phenol extracted (21)
and quantitated by 260 nm absorbance. Protein and RNA contamination was checked through the $A_{260}/A_{280}$ ratio.

Production of the Antiserum. The anti-2-AAF immune serum was produced by immunization of adult New Zealand white rabbits with six weekly subcutaneous injections of 2-AAF–gelatin (1 mg) emulsified in complete Freund’s adjuvant (0.5 ml). A 2 mg booster injection was delivered 15 days after the last inoculation. One week later, the rabbits were bled from the heart.

Enzyme-linked Immunoabsorbent Assays (ELISA). For the direct version of the assay, polystyrene 96-well microtiter plates were coated with 2-AAF–gelatin conjugate (containing 0.05–0.5 ng 2-AAF). Then, 50 µl of rabbit anti-2-AAF antiserum, diluted 2,500-fold, was added to each well. Goat anti-rabbit immunoglobulin G (IgG)–horseradish peroxidase conjugate (Bio-Rad, Hercules, CA) was used 1:2,000 as the second antibody with o-phenylenediamine as the substrate (22).

DNA–2-AAF adducts were measured by competitive ELISA (23,24). Briefly, 96 wells were coated with 0.25 ng 2-AAF–gelatin and then saturated with 200 µl of 0.5% gelatin. Standard curves were obtained by adding to each well 50 µl of rabbit antiserum diluted 1:5,000 that had been preincubated 2 hr with serial dilutions of 2-AAF–gelatin as a competitor. For 2-AAF–DNA adducts determination, 1.5 µg of DNA was used as competitor. Goat anti-rabbit IgG–horseradish peroxidase conjugate was used as the second antibody diluted 1:4,000, with o-phenylenediamine as substrate.

Results and Discussion

To elicit a humoral response toward 2-AAF, mice received repeated ip injections of a complete immunogen, as detailed in the previous section. Due to the low molecular weight of the hapten (insufficient to produce an effective immunogenic stimulus) a carrier molecule (gelatin) was conjugated to 2-AAF. At the end of immunization, the presence of specific IgG directed against the 2-AAF–gelatin conjugate in the sera of immunized mice was assessed by direct ELISA. Although interindividual differences in serum titers were observed, all the immunized mice examined produced humoral antibodies capable of reacting against the 2-AAF conjugate (Figure 1). Conversely, serum pools of both nonimmunized mice and mice treated with the adjuvant alone did not show any reactivity toward 2-AAF–gelatin. In all cases, no reactivity was observed against the carrier protein alone (data not shown).

2-AAF–DNA binding in livers of both immunized and nonimmunized mice was determined by competitive ELISA using a polyclonal rabbit antiserum. This serum was able to recognize the carcinogen adducted to gelatin and bovine, egg, or mouse serum albumin (Figure 2). Similar results were obtained with pooled sera of immunized mice (Figure 2). In both cases, no reactivity was observed with the carrier proteins alone (data not shown). As the reactivity of rabbit serum toward DNA and protein adducts by competitive ELISA was basically similar (Figure 3), the gelatin conjugate was used as quantitative standard in all determinations.

The quantitation of liver DNA adducts after 4 weeks of dietary exposure to 2-AAF demonstrated high levels of adducts in all treated animals, with a partial quantitative relationship with the administered dose (Table 1). No reactivity was observed with DNA of untreated mice (100% of reactivity in competitive ELISA), which was used as an internal experimental control. A comparison of the results obtained with immunized and nonimmunized mice (receiving either the vehicle or the adjuvant alone) revealed a highly significant difference among groups, with far lower adduct levels in immunized mice (Table 1). No significant differences in food consumption were observed between immunized and nonimmunized mice, suggesting that all animals received comparable amounts of carcinogen at the end of treatment. Across all the experimental groups, liver weights were fairly similar; this suggested the

### Table 1. Liver DNA adducts in mice following 4 weeks dietary exposure to 2-AAF.

| Treatment (ppm) | None       | Adjuvant | Adjuvant and immunogen |
|----------------|------------|----------|------------------------|
|                |            |          |                        |
| 50             | 101±9 (14) | 115±13 (11) | 47±13 (9)*            |
| 150            | 245±26 (14) | 182±19 (12)** | 62±13 (12)*          |

Liver DNA adducts shown in fmol 2-AAF/µg DNA (mean±SE). Value in parentheses $= n$. Statistical significance of differences observed among experimental groups was evaluated by Student’s t-test: *$p<0.001$ (vs nonimmunized) and $p<0.01$ (vs adjuvant alone); **$p<0.05$ (vs nonimmunized); $p<0.001$ (vs both nonimmunized and adjuvant alone groups).
absence of significant liver toxicity. The possible indirect effect of immunization, based on the impairment of liver enzymatic activities, was investigated in satellite groups of immunized and nonimmunized mice using liver homogenates for the exogenous activation of 2-AAP to mutagen in the Salmonella reversion assay. Almost identical results were obtained with homogenates from different experimental groups (data not shown), indicating that the immunization pretreatment did not exert any significant detrimental effect on these liver activities.

The mechanism underlying the intriguing result provided by this study is not yet clear. As stated above, trivial bias due to a perturbatory effect of the immunization on liver function cannot be ruled out. Organ weight, DNA, and protein content, and efficiency in the activation of 2-AAP to bacterial mutagen were fairly similar in immunized and nonimmunized mice. An unspecific perturbation related to the stimulation of the immune system is also unlikely in view of the results obtained with mice treated with the Freund’s adjuvant alone; these mice experienced a significant yet unspecific immunogenic stimulation. Therefore, alternative mechanisms have to be considered. One plausible explanation lies in the high reactivity of the specific antibodies elicited by the immunization toward a variety of carcinogen conjugates. Considering that hydrophobic carcinogens are largely present in the blood stream in association with carrier proteins, it is conceivable that circulating antibodies may act as scavengers that effectively lower the bioavailable fraction of the compound entering into the organism.

It can be supposed that the production of a specific humoral immunological response after chronic carcinogen exposure may be a widespread phenomenon. Production of specific antibodies has been observed in mice (27) and in humans (28,29) with prolonged exposure to DNA-damaging agents, suggesting that chronic adduct formation on serum proteins or DNA can fulfill the steric and structural conditions required for the induction of an immune humoral response. In any case, no definitive conclusion should be drawn on the biological significance of the phenomenon described herein. Further studies are required to investigate its specificity, as well as the profile of damage induced in other target tissues. Nevertheless, the data presented support the hypothesis that the immunological response induced by chronic exposures may act as a significant host factor in environmental carcinogenesis.

REFERENCES

1. Cleaver JE. Defective DNA repair and cancer-prone disorders of man. In: Genes and Cancer (Bishop JM, Rowley JS, Grees M, eds). New York:Alan Liss, 1984; 17–135.
2. Friedberg EC. ed. DNA damage and human disease. In: DNA Repair. New York:WH Freeman, 1985; 505–574.
3. Lehmann AR, Dean SW. Cancer-prone human disorders with defects in DNA repair. In: Chemical Carcinogenesis and Mutagenesis, Vol II (Cooper CS, Grover PL, eds). Berlin: Springer-Verlag, 1990:71–101.
4. Cartwright RA, Glashan RW, Rogers HJ, Ahmad RA, Barham-Hall D, Higgins E, Kahn MA. Role of N-acetyltransferase phenotypes in bladder carcinogenesis: a pharmacogenetic epidemiological approach to bladder cancer. Lancet 2: 842–845 (1982).
5. Vahakangas K, Aputur H, Harris CC. Interindividual variation in carcinogen metabolism, DNA damage and DNA repair. In: Monitoring Human Exposure to Carcinogenic and Mutagenic Agents (Berlin A, Draper M, Hemminki K, Vainio H, eds). IARC Scientific Publications No. 59. Lyon:International Agency for Research on Cancer, 1983: 85–98.
6. Caporaso NE, Tucker MA, Hover RN, Hayes RB, Pickle LW, Issaq HJ, Muschik GM, Green-Gallo L, Buysys D, Aisner S, Resau JH, Trump BF, Tollerud D, Weston A, Harris CC. Lung cancer and the debrisoquine metabolic phenotype. J Natl Cancer Inst 82:1264–1272 (1990).
7. Conney AH. Induction of microsomal enzymes by foreign chemicals and carcinogenesis by polycyclic aromatic hydrocarbons: G.H.A. Clowes Memorial Lecture. Cancer Res. 42: 4875–4917 (1982).
8. Kej TJ, Beral V. Sex hormones and cancer. In: Mechanisms of Carcinogenesis in Risk Identification (Vainio H, Magee P, McGregor D, Michael AJ, eds). IARC Scientific Publications No. 116. Lyon:International Agency for Research on Cancer, 1992:255–269.
9. Klinef J. Immunosuppression and cancer. In: Mechanisms of Carcinogenesis in Risk Identification (Vainio H, Magee P, McGregor D, Michael AJ, eds). IARC Scientific Publications No. 116. Lyon:International Agency for Research on Cancer, 1992:237–253.
10. Sanders BM, Jay M, Draper GJ, Roberts EM. Non-ocular cancer in relatives of retinoblastoma patients. Br J Cancer 60:358–365 (1989).
11. Srivastava S, Zou ZQ, Pirollo K, Blattner W, Chang EH. Germ line transmission of a mutated p53 gene in a cancer-prone family with Li-Fraumeni syndrome. Nature 348:747–749 (1990).
12. Harris CC, Mulvihill JJ, Thorgerisson SS, Minna JD. Individual differences in cancer susceptibility. Ann Intern Med 92:809–825 (1980).
13. Harris CC. Concluding remarks: role of carcinogens, cocarcinogens and host factors in cancer risk. In: Human Carcinogenesis (Harris CC, Aputur H, eds). New York:Academic Press, 1983:941–970.
14. Perera FP, Weinstein IB. Molecular epidemiology and carcinogenesis—DNA adduct detection: new approaches to studies of human cancer causation. J Chronic Dis 35:581–600 (1982).
15. Harris CC, Vahakangas K, Newman MJ, Trivers GE, Shamsuddin A, Sinopoli N, Mann DL, Wright WE. Detection of benzo(a)pyrene diol-epoxide—DNA adducts in peripheral blood lymphocytes and antibodies to the adducts in serum from coke oven workers. Proc Natl Acad Sci USA 82:6672–6676 (1985).
16. Haugen A, Becher G, Benstad C, Vahakangas K, Trivers GE, Newman MJ, Harris CC. Determination of polycyclic aromatic hydrocarbons in the urine, benzo(a)pyrene diol-epoxide—DNA adducts in lymphocyte DNA, antibodies to the adducts in sera from coke-oven workers exposed to measured amounts of polycyclic aromatic hydrocarbons in the work atmosphere. Cancer Res 46:4178–4183 (1986).
17. Newman MJ, Light BA, Weston A, Tollerud D, Clark JL, Mann DL, Blackmon JP Harris CC. Detection and characterization of human serum antibodies to polycyclic aromatic hydrocarbon diol-epoxide DNA adducts. J Clin Invest 82:145–193 (1988).
18. Wogan GN. Methods. In: Methods for Detecting DNA Damaging Agents in Humans: Application in Cancer Epidemiology and Prevention (Bartsch H, Hemminki K, O'Neill IK, eds). IARC Scientific Publications No. 89. Lyon:International Agency for Research on Cancer, 1988:9–12.
19. Silibart JK, Keren DF. Reduction of intestinal carcinogen absorption by carcinogen-specific secretory immunity. Science 243:1462–1464 (1989).
20. Verdina A, Zito R. Substitute anilines as haptens. J Exp Clin Cancer Res 14:39-43 (1995).
21. Sambrook J, Fritsch EF, Maniatis T. Molecular Cloning. A Laboratory Manual. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press, 1989.
22. Santella MR, Dharmanaja N, Gasparro FP, Edelson RL. Monoclonal antibodies to DNA modified by 8-methoxypsoralen and ultraviolet A light. Nucleic Acid Res 13:2533-2544 (1985).
23. Green N, Alexander H, Olson A, Alexander R, Shinnick TM, Sutcliffe JG, Lerner RA. Immunogenic structure of the influenza virus hemagglutinin. Cell 28:477-487 (1982).
24. Citro G, Verdina A, Galari R, Floris G, Sabatini S, Finazzi-Agro' A. Production of the antibodies against the coenzyme pyrrolequinoline quinone. FEBS Lett 247:210-214 (1989).
25. Shu HP, Bymun EN. Systemic excretion of benzo(a)pyrene in the control and microsomally induced rat: the influence of plasma lipoproteins and albumin as carrier molecules. Cancer Res 43:485-490 (1983).
26. Arif JM, Gupta RC. Detection of DNA-reactive metabolites in the serum and their distribution in rats exposed to artificial carcinogen mixture. Proc Am Assoc Cancer Res 36:151 (1995).
27. Lee BM, Strickland PT. Antibodies to carcinogen-DNA adducts in mice chronically exposed to polycyclic aromatic hydrocarbons. Immunol Lett 36:117-123 (1993).
28. Dubroff LM, Reid RJ Jr. Hydralazine-pyrimidine interactions may explain hydralazine-induced lupus erythematosus. Science 208:404-406 (1980).
29. Reidenberg MM, Drayer DE. Aromatic amines and hydrazines, drug acetylation, and lupus erythematosides. Human Genet Suppl 1:57-63 (1978).