Effect of Sodium Fluoride on Antibody Formation in Rabbits

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In the present study, the role of sodium fluoride on antibody formation in rabbits is assessed. Sixteen female albino rabbits were divided into four groups and were treated as follows: Group I: Animals immunized with transferrin and used as controls for group II; Group II: Animals immunized with transferrin and administered orally NaF (10 mg/kg body weight) daily for 9 months; Group III: Animals immunized with transferrin and served as controls for group IV; and Group IV: Animals administered with NaF at a dose of 10 mg/kg body weight daily for 9 months and then immunized with transferrin and fluoride treatment continued for another 9 months. Rabbits were bled just prior to immunization and subsequent bleeding was at weekly intervals. Circulating anti-transferrin titers were estimated. DNA and protein synthesis was determined by incorporating [^{3}H]thymidine and [^{14}C]leucine, respectively. The present report demonstrates that sodium fluoride inhibits antibody formation in rabbits. There is a threshold level of fluoride (0.78 ppm) in circulation which is responsible for the inhibitory effect on antibody formation. Inhibition of DNA and protein synthesis by NaF is also demonstrated. It is concluded that fluoride inhibits the antibody formation by decreasing the proliferation of lymphocytes and by inhibiting the protein synthetic ability of immunocytes. © 1987 Academic Press, Inc.

INTRODUCTION

Fluoride is known to induce hematological alterations in rabbits when ingested in excess over a prolonged period of time (Hirao, 1972; Susheela and Jain, 1983). According to Susheela and Jain, fluoride influences the alterations in blood cell counts including that of lymphocytes, possibly due to adrenal cortical malfunction. It is known that adrenal cortex has an influence on the normal structure and function of the lymphoid tissue (White *et al.,* 1978).

Berry and Trillwood (1963) have shown that the cytotoxic effect of fluoride inhibits cell growth and function. Other reports with similar conclusions were later confirmed by Albright (1964), Hongslo *et al.* (1974), and Imai *et al.* (1983). Sodium fluoride also inhibits DNA synthesis in isolated nuclei (Proffit and Ackerman, 1964). However, Imai *et al.* (1983) have reported that sodium fluoride inhibits DNA synthesis by making unavailable the protein(s) required for it. It is thus possible that inhibitory effect of fluoride on DNA synthesis is a result of its inhibition of protein synthesis.

The decrease in lymphocyte population, adrenal cortical malfunction, and defective protein biosynthesis may have an influence on the immune status of the animal. In the present study, the role of sodium fluoride on the antibody formation in rabbits has therefore been explored.

MATERIALS AND METHODS

Sixteen female albino rabbits weighing 300–400 g maintained on standard diet

(Hindustan Lever) and water *ad libitum* were kept under constant laboratory conditions and divided into the following four groups of four animals each.

> Group I: Animals immunized with transferrin and used as controls for group II animals.
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> Group II: Animals immunized with transferrin and administered orally with NaF (10 mg/kg body weight) daily for 9 months.
>
> Group III: Animals immunized with transferrin and served as controls for group IV animals.
>
> Group IV: Animals administered with NaF (10 mg/kg body weight) daily for 9 months and thereafter immunized with transferrin and fluoride treatment continued for another 9 months.

Immunization

All animals were immunized with transferrin (Tf, 20 μg/kg body weight) with complete Freund's Adjuvent (CFA). A primary booster of antigen was given after 5 to 6 weeks of immunization with incomplete Freund's Adjuvent (IFA). Rabbits were bled just prior to immunization and subsequent bleeding was at weekly intervals. A secondary booster of antigen (Tf, 20 μg/kg body weight) with IFA was given to all animals when circulating anti-transferrin antibodies (anti-Tf titers) started decreasing. The animals showing low circulating anti-Tf titers, i.e., less than 10% binding at 1:4000 dilution, were dropped from further studies.

Circulating Levels of Anti-Tf Antibody

Anti-Tf titers in circulation were estimated from the serum by RIA using ^{131}I-labeled transferrin. The assay mixture contained 50 μl of diluted serum, 50 μl of ^{131}I-labeled transferrin (10,000 counts approx.), and 50 μl of RIA buffer (pH 7.4). After vortexing, the assay mixture was incubated at 4°C for 16–20 hr. In order to decrease the nonspecific binding, the final volume of assay mixture after incubation was made to 300 μl with RIA buffer and then precipitated with 300 μl of 20% polyethylene glycol (PEG). Percentage binding was calculated at different dilutions.

DNA and Protein Synthesis

DNA and protein synthesis was determined by the incorporation of [^{3}H]thymidine and [^{14}C]leucine respectively into peripheral human lymphocytes. Lymphocytes were separated from the peripheral venous blood using Ficoll–Hipaque medium and were washed with Eagle's minimum essential medium (EMEM) under sterile conditions. One million cells in each well were seeded into a 24-well culture plate in EMEM medium containing 10% fetal calf serum. The optimum concentration of phytohemagglutinin (PHA) (40 μg/well, earlier standardized in laboratory) was added to each well simultaneously. Twenty-four hours after seeding of cells, sodium fluoride was added to culture medium at different concentrations: 0, 2, 3, 4, 5, 10, and 20 mM in different wells. Cells were in their logarithmic phase at this time.

[³H]Thymidine Uptake

The cultures were exposed to 2.5 μCi per well of [³H]thymidine (specific activity 21 Ci/mmole; The Radiochemical Centre, Amersham) after 48 hr of seeding. The cells were harvested after 72 hr of seeding by precipitating over Whatman No. 1 filter paper with 5% cold trichloroacetic acid and cold methanol. Filters were dried and counted in a Packard Tri-Carb liquid scintillation counter with toluene-based scintillation fluid.

[¹⁴C]Leucine Uptake

[¹⁴C]Leucine (0.3 μCi, specific activity 342 mCi/mmole; The Radiochemical Centre, Amersham) was added to each culture well 6 hr before harvesting. After the incubation period was over, cells were harvested as described above and counted. The number of viable lymphocytes after every 24 hr of culture was counted by the trypan blue exclusion method as described by Winchester and Ross (1976).

Fluoride Estimation

Fluoride from serum was determined using the method of Hall *et al.* (1972).

RESULTS

Status of Fluoride in Circulation after Treatment

The levels of fluoride in serum of animals of groups I and II are shown in Fig. 1. The results indicate that in animals of group II a plateau in serum concentration of fluoride is reached after the 24th week of treatment which is maintained until
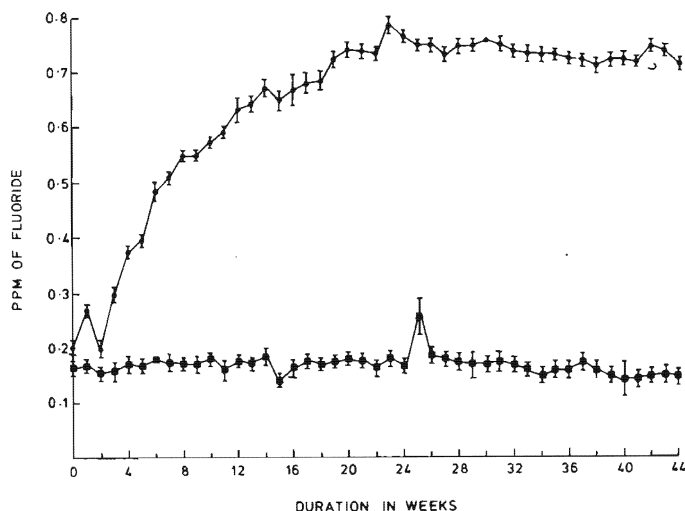

FIG. 1. The serum fluoride levels in ppm in normal animals of group I (■) and NaF-treated animals of group II (●). Values are expressed as mean ± SD. A plateau in serum concentration of fluoride is reached after the 24th week of treatment which is maintained until the 44th week.
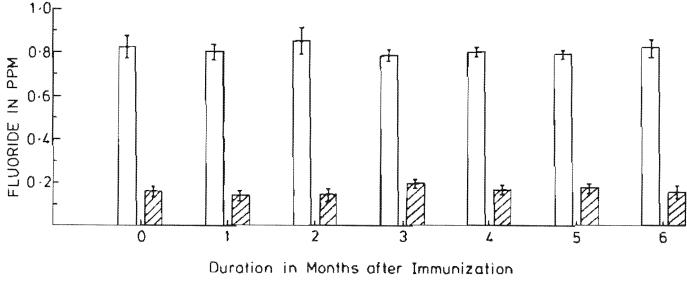
FIG. 2. The serum fluoride levels in ppm in control animals of group III (hatched) and 9-month NaF-treated rabbits of group IV (open). Values are expressed as mean ± SD. The concentration of serum fluoride which was reached at the time of immunization in animals of group IV was maintained throughout the experimental period.

the 44th week. In Fig. 2, the results on serum fluoride levels in control animals of group III and 9-month NaF-treated rabbits of group IV are shown. The concentration of serum fluoride at the time of immunization of animals of group IV is maintained throughout the experimental period.

Effect of Sodium Fluoride on Antibody Formation against Transferrin

The anti-Tf titers in animals of groups I to IV are shown in Figs. 3–6. It is evident from the results that the animals of groups I, II, and III showed (Figs. 3, 4, and 5) elevated antibody titers 2 weeks after the primary booster. Anti-Tf titers in group II (Fig. 4) were decreased after 20 to 24 weeks of immunization and did not show any significant elevation following a secondary booster, while the titers of antibodies were significantly elevated in animals of group I (Fig. 3) after the secondary booster when compared to the experimental group (group II; Fig. 4). To determine whether this change in response to antibody formation is because of simultaneous ingestion of fluoride or a cumulative effect of fluoride, two groups
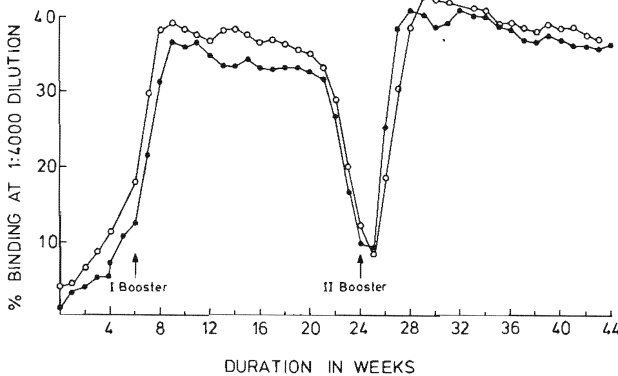

FIG. 3. Circulating anti-Tf titers in control rabbits of group I. Curves are plotted using percentage binding at 1:4000 serum dilution versus time interval in weeks for individual animals. Anti-Tf titers shot up after the primary booster was given at the 6th week and again after giving the secondary booster at the 24th week after immunization in both animals. (●) Animal 1; (○) animal 2.
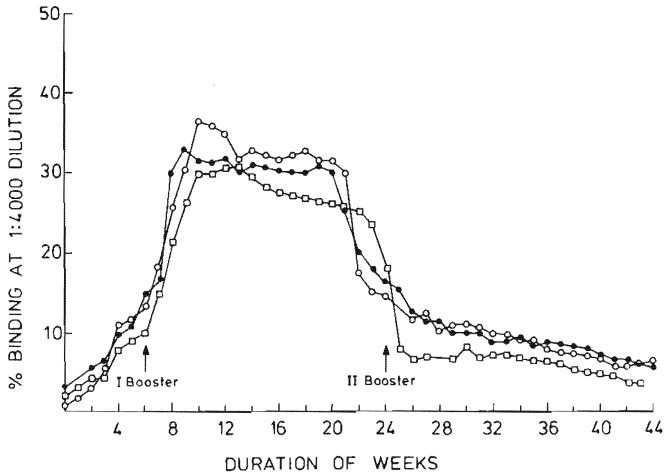
FIG. 4. Circulating anti-Tf titers in NaF-treated rabbits of group II. Values are plotted using percentage binding at 1:4000 serum dilution versus time interval in weeks for individual animals. There is no change in anti-Tf titers after the secondary booster. (●) Animal 1; (○) animal 2; (■) animal 3.

of animals (groups III and IV) were further immunized with transferrin. Anti-Tf titers were estimated and results are shown in Figs. 5 and 6. The animals of group IV (Fig. 6) had high circulating levels of fluoride at the time of immunization. Animals in group III served as sex- and age-matched controls for group IV. Re-
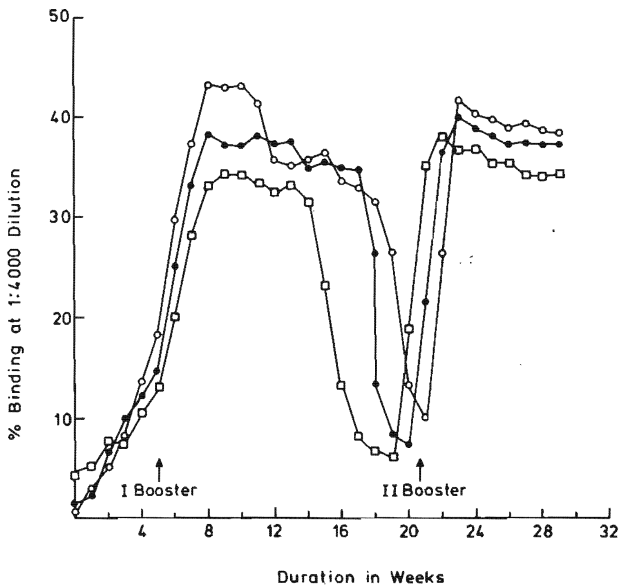

FIG. 5. Circulating anti-Tf titers in control rabbits of group III. Values are plotted using percentage binding at 1:4000 serum dilution versus time interval in weeks for individual animals. Anti-Tf titers shot up after giving the primary booster at the 5th week and again when the secondary booster was given at the 20th week after immunization.
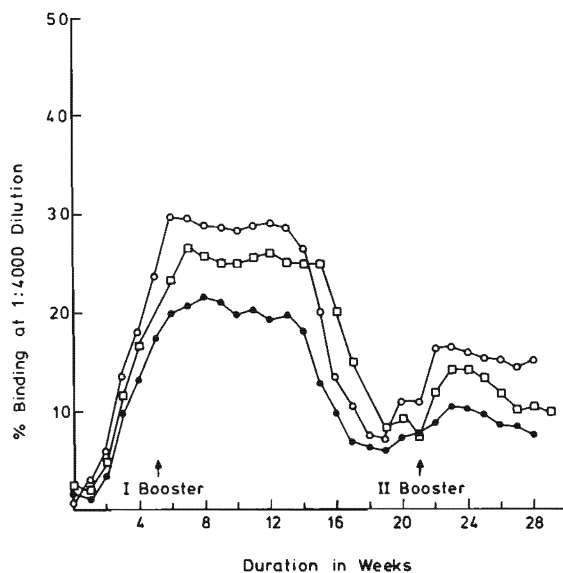
FIG. 6. Circulating anti-Tf titers in NaF-treated rabbits of group IV. Values are plotted using percentage binding at 1:4000 serum dilution versus time interval in weeks for individual animals. There is a very weak response to the secondary booster on anti-Tf titers.

sults in Fig. 6 and Table 1 indicate that the titers of antibodies were significantly low ($P < 0.01$) in animals of group IV after immunization when compared to their corresponding controls of group III (Fig. 5) and to the animals of groups I and II. The animals of group III showed a significant elevation in titers of antibodies after the secondary booster while the animals of group IV showed a very weak response to the secondary booster.

Effect of Sodium Fluoride on DNA Synthesis

It is evident that the accumulation of fluoride in circulation in rabbits over a prolonged period causes an alteration in response to antibody formation. In order to explore the possible cause for this effect, DNA synthesis in cultured lymphocytes was studied by measuring the uptake of radioactive DNA precursor, [3H]thymidine, 24 hr after the addition of different concentrations of sodium fluoride (0, 2, 3, 4, 5, 10, and 20 mM) into culture medium. The results shown in Fig. 7 reveal the incorporation of 77, 71, and 70% of [3H]thymidine in cultures containing 2, 3, and 4 mM of NaF, respectively. The uptake of thymidine is inhibited drastically to 88, 91, and 95% in cultures containing 5, 10, and 20 mM of NaF, respectively. The results obtained indicate that the cellular DNA synthesis is inhibited from 0 to 95% by addition of various concentrations (0–20 mM) of NaF.

Effect of Sodium Fluoride on Protein Synthesis

The results obtained on the effect of sodium fluoride on protein synthesis by [14C]leucine uptake in cultured lymphocytes are shown in Fig. 8 and are similar to those of DNA synthesis. It is therefore concluded that sodium fluoride inhibits protein synthesis.
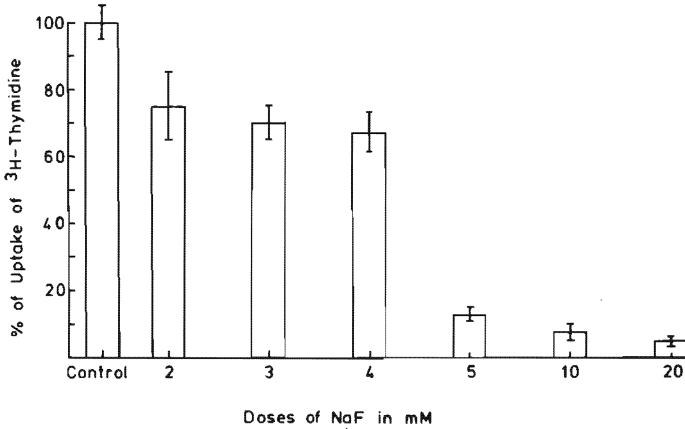
FIG. 7. The effect of various concentrations of sodium fluoride on DNA synthesis. [³H]Thymidine incorporation in human lymphocytes is shown (mean ± SD), expressed as percentage of thymidine incorporation (with respect to control, 0.0 mM NaF). Sodium fluoride (5, 10, and 20 mM) -containing cultures showed a highly significant reduction of [³H]thymidine incorporation.

Effect of Fluoride on Cell Viability

The observed inhibition of DNA and protein synthesis by sodium fluoride in cultured lymphocytes may be due to its toxic effect on cells. The viability of cells in culture after every 24 hr up to a maximum of 96 hr was checked and results are shown in Table 2. It is evident from the results that 98 to 78% of the cells are viable throughout the culture period in the presence of NaF at concentrations ranging from 2–5 to 10 mM. However, only 38% of cells were observed to be viable at 20 mM of NaF in culture medium after 72 hr. Hence, it is concluded that the presence of 20 mM of NaF in culture medium is sublethal.
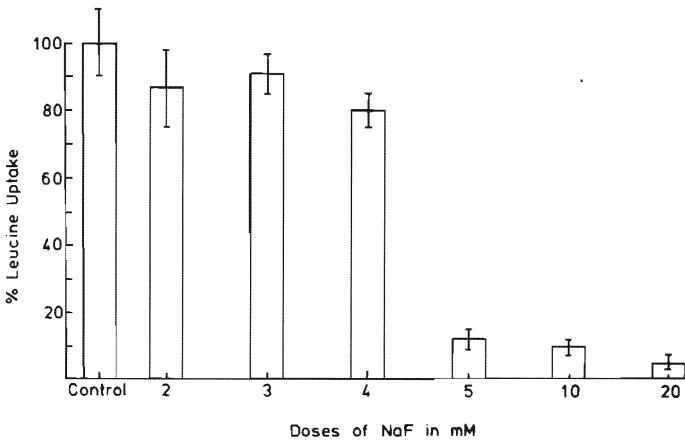

FIG. 8. The effect of various concentrations of sodium fluoride on protein synthesis. [¹⁴C]Leucine incorporation in human lymphocytes is shown (mean ± SD), expressed as percentage of leucine incorporation (with respect to control, 0.0 mM NaF). Sodium fluoride (5, 10, and 20 mM) -containing cultures showed a highly significant reduction in [¹⁴C]leucine incorporation.

tion in lymphocytes may be due to the inhibition of lymphocyte proliferation and hence the reduction of the antibody formation in fluoride-treated rabbits.

The reduction in antibody titers in rabbits after 6 months of fluoride ingestion or in rabbits treated with sodium fluoride for 9 months prior to immunization is further attributed to the fact that fluoride would have inhibited the protein synthetic ability of the antibody-forming cells, i.e., the immunocytes. Fluoride is also known to inhibit the protein synthesis under *in vivo* conditions in rabbits (Kathpalia and Susheela, 1978). It is therefore concluded that after prolonged exposure to NaF, the circulating antibody titers in rabbits are reduced, which might adversely affect the defense mechanism.

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