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Immunology

Antigen stimulation of prostaglandin synthesis and control of immune responses

(humoral response/cell-mediated response)

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ABSTRACT **Within 2 min following the intravenous injection of sheep erythrocytes (sRBC) there occurs 20 to 80-fold increase in prostaglandin (PG) $F_{2\alpha}$ in the spleen. This burst of synthesis is followed by a slow decline to control levels over the next 1–4 hr. No increase in splenic $PGF_{2\alpha}$ levels is observed between 24 and 72 hr after injection. Injection of colloidal carbon results in a small increase, approximately 20% of the increase in $PGF_{2\alpha}$ observed with sRBC. The early increase in splenic $PGF_{2\alpha}$ levels stimulated by sRBC is also dependent upon thymus-derived (T) cells, since the increase is small or nonexistent in athymic mice and NZB mice. Also, the elevation of splenic $PGF_{2\alpha}$ levels is blocked by the administration of indomethacin or Ro 20-5720, both of which block the synthesis of prostaglandin. A small increase (2-fold) in $PGF_{2\alpha}$ levels occurs in the thymus. A soluble antigen, bovine gamma globulin, stimulated a bimodal increase in splenic $PGF_{2\alpha}$ levels, the early peak occurring at 2 hr and the later increase occurring at 48 hr. Using inhibitors of prostaglandin synthesis, it is possible to enhance the appearance of cells forming 19S antibody against sRBC, both *in vivo* and *in vitro*. Furthermore, inhibition of prostaglandin synthesis enhances DNA synthesis induced in a two-way mixed-lymphocyte reaction only in whole spleen cell cultures and not in cultures of spleen cells purified by passage over glass wool. Based on this evidence, it is proposed that the prostaglandins represent a major soluble mediator in the control of T cell–T cell interactions and also play an important part in T–B (bone-marrow derived) cell interactions.**

The stimulation of lymphocytes by specific and nonspecific agents (antigens or mitogens) leads to a complex series of events culminating either in the development of an immune response or in the expression of a variety of immunological functions (1, 2). Over the past few years, the attempts at identifying specific products formed by stimulated lymphocytes have been only marginally successful (3). An alternative, namely, the study of the effects of known, pharmacologically active substances on immunocompetent cells has also been done (4–8). It is now possible to identify several kinds of compounds which affect various aspects of lymphocyte function. Recently, in our laboratory, it was demonstrated that sheep erythrocytes, when injected intravenously, caused very large changes in splenic prostaglandin levels very quickly following injection. The increase was shown to be dependent on the presence of thymus-derived (T) lymphocytes (9). Based on this and the evidence from other laboratories that mitogens may also induce increases in prostaglandin levels *in vitro* (10), the present investigation was undertaken to explore the role of prostaglandin synthesis in the regulation and control of immune responses.

Abbreviations: T cell, thymus-derived cell; sRBC, sheep erythrocytes; BGG, bovine gamma globulin; PG, prostaglandin; MLR, mixed lymphocyte reaction; NAL, nonadherent (to glass wool) lymphocytes.

MATERIALS AND METHODS

Animals. C57Bl/6J and C_3H/HeJ male and female mice, 6–10 weeks old, were purchased from Jackson Laboratories (Bar Harbor, Maine). The NZB mice were kindly provided by Dr. Norman Talal of the University of California (San Francisco).

Culture Techniques. For *in vitro* antibody formation, the culture method of Mishell and Dutton (11, *), as modified by Click *et al.* (12) was used. Measurement of direct (19 S) antibody-forming cells was carried out using localized hemolysis in gel according to the method of Jerne *et al.* (13).

Mixed lymphocyte reactions (MLR) were performed by mixing various proportions of C57Bl/6J and C_3H/HeJ spleen cells together in microtiter wells. The culture medium used was RPMI 1640 with glutamine and penicillin–streptomycin added; there was no serum supplement. Separation of spleen cells for the MLR was performed using glass wool columns as outlined by Folch and Waksman (14) and modified for use with mouse spleen cells as previously reported†. DNA synthesis in the MLR cultures was measured from the incorporation of [^{3}H]thymidine ([^{3}H]dThd, Amersham/Searle, Chicago, Ill.) into trichloroacetic-acid-precipitable material. Cultures in the microtiter wells were harvested using a multiple automated sample harvester (MASH II, Microbiological Associates, Bethesda, Md.) according to the method of Hartzman *et al.* (15).

Antigens. Sheep erythrocytes (sRBC) from a single sheep, stored in Alsevers solution at 4°, were purchased from Colorado Serum Co. (Denver, Colo.). The sRBC were washed three times in saline prior to injection. Bovine gamma globulin (BGG) was obtained from Sigma (St. Louis, Mo.), and stored at −20° until use.

Prostaglandin Inhibitors. Indomethacin, an irreversible, noncompetitive inhibitor of prostaglandin synthesis was purchased from Sigma. Octadeca-9,12-diynoic acid (Ro 3-1314), an irreversible inhibitor, and *d,l*-6-chloro-α-methyl-carbazole-2-acetic acid (Ro 20-5720), a competitive inhibitor of prostaglandin synthesis, were a gift from Hoffmann-La-Roche, Inc. (Nutley, N.J.).

Measurement of Prostaglandin. The assay of splenic prostaglandin (PG) $F_{2\alpha}$ levels was carried out using a radioimmunoassay as previously described (9, 16, 17).

RESULTS

Effects of sRBC on Splenic PG Levels. In a preliminary report from this laboratory (9), it was established that sRBC, injected intravenously, could stimulate a rapid increase in the level of $PGF_{2\alpha}$ in the mouse spleen within 2 min after

* A. T. Jamieson and D. R. Webb, submitted for publication.
† D. R. Webb and A. T. Jamieson, submitted for publication.

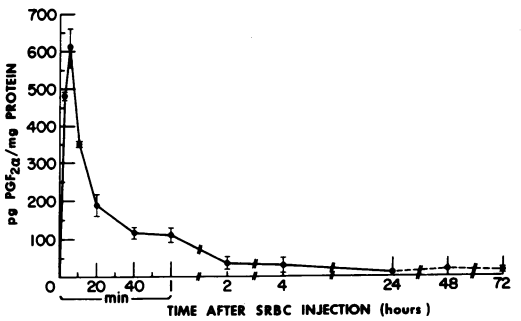

FIG. 1. Changes in splenic $PGF_{2\alpha}$ levels following stimulation *in vivo* by sRBC. C57Bl/6J mice were injected with 2×10^8 sRBC i.v.; control mice received saline. Each point represents the mean value obtained using three mice, ± standard error of the mean. The control PG level was 31.7 ± 6.8 pg/mg of protein.

injection. It was also established that this increase was dependent on the presence of T cells. The experiment depicted in Fig. 1 confirms and extends this observation to include measurements of spleen $PGF_{2\alpha}$ levels over several days. Following the initial 20-fold increase in PG levels, there is a decline in $PGF_{2\alpha}$ levels, which then remain slightly above the control value for several hours. Between 24 and 48 hr $PGF_{2\alpha}$ levels remain at control levels. Table 1 lists the effects of various doses of sRBC on splenic $PGF_{2\alpha}$ levels. The level of splenic PG measured at 2 min is still increasing at a dose of 10^9 sRBC (packed cells) (36.5-fold over control). In the experiment shown in Table 1 (Part B), the effects of colloidal carbon were also tested in order to examine the possible role of phagocytosis or clearance in the induction of splenic PG. The data show that, in fact, a portion (approximately 20% of that observed when compared to several experiments using sRBC) of the increase at 2 min is attributable to a nonspecific clearance effect.

These data demonstrate the capacity of a particulate antigen to induce rapid, large increases in PG levels. It is important, therefore, to determine whether compounds which are known to block PG synthesis prevent the early changes shown here. The experiment presented in Table 2 demonstrates the effects of two inhibitors of PG synthesis on the sRBC-induced increase in splenic PG levels. Both indomethacin, a noncompetitive irreversible PG synthesis inhibitor, and Ro 20-5720, a reversible, competitive inhibitor, block the early increase in $PGF_{2\alpha}$ induced by sRBC.

Table 1. Effect of various doses of sRBC and colloidal carbon on splenic $PGF_{2\alpha}$ levels

Injection	$PGF_{2\alpha}$*, pg/mg of splenic protein
A.	
Saline control	26.4 ± 1.62
sRBC, 10^6/mouse	31.5 ± 6.59
10^7/mouse	109.6 ± 30.0
10^8/mouse	654.1 ± 155.7
5×10^8/mouse	865.7 ± 123.7
10^9/mouse	953.5 ± 98.6
B.	
Saline control	26.4 ± 1.62
Carbon†	225.2 ± 46.1
sRBC, 2×10^8/mouse	707.2 ± 55.0

* $PGF_{2\alpha}$ levels were measured in splenic extracts 2 min after injection.

† The colloidal carbon mixture consisted of 1.5 ml of Pelikan ink, 3.0 ml of saline, and 0.5 ml of 0.6% gelatin in saline. 0.2 ml per mouse was injected i.v.

Table 2. Ro 20-5720 and indomethacin block the increase of splenic $PGF_{2\alpha}$ levels in C57 mice injected with sRBC

Inhibitor	$PGF_{2\alpha}$, pg/mg of protein*		
	Control	2 min	5 min
None	43.4 ± 1.15	707.3 ± 45.5	400.9 ± 37.8
Ro 20-5720†	35.2 ± 0.43	102.8 ± 30.7	72.3 ± 18.7
Indomethacin†	28.2 ± 2.34	28.8 ± 0.5	—

* Values are given ± standard error of the mean.

† Ro 20-5720 and indomethacin were injected i.p. −24, −2, and 0 hr relative to the time of sRBC injection. In all experiments 2×10^8 sRBC were injected i.v. and animals were sacrificed 2 and 5 min after sRBC injection; control received saline (0.2 ml).

Effects of sRBC on Thymic PG Levels and on Splenic PG Levels in NZB Mice. Since it has previously been established that the elevation of splenic PG levels is dependent on the presence of T cells, experiments were carried out to further identify the nature of the cell type(s) involved which respond to antigen by synthesizing PG. In the experiment shown in Fig. 2, the levels of $PGF_{2\alpha}$ in the thymus were measured following intravenous antigen administration. It should first be noted that basal levels in unstimulated thymus are approximately twice those normally seen in the spleen. Second, an increase in $PGF_{2\alpha}$ occurs at about 20 min; it is much smaller (2-fold) than that observed in the normal spleen (approximately 20 to 80-fold) or that seen in the spleens of athymic nude mice (approximately 10-fold) (9). Since the thymus lacks the blood supply which is present in the spleen, it is doubtful that this increase is due to contact between antigen-sensitive thymocytes and sRBC.

Once it had been established that the greatest change in PG occurs in the spleen and is dependent on the T cells found there, further experiments were performed using another strain of mice in which a defect in thymus cell development exists. Several laboratories (18, 19) have demonstrated that the NZB mouse, which develops spontaneous autoimmune disease (20) is deficient in T suppressor cell activity. Accordingly, young NZB mice (5 weeks old), which still possess suppressor function, and old NZB mice (1 year old), in which suppressor function is lacking, were injected with sRBC and the levels of splenic PG were measured. The re-

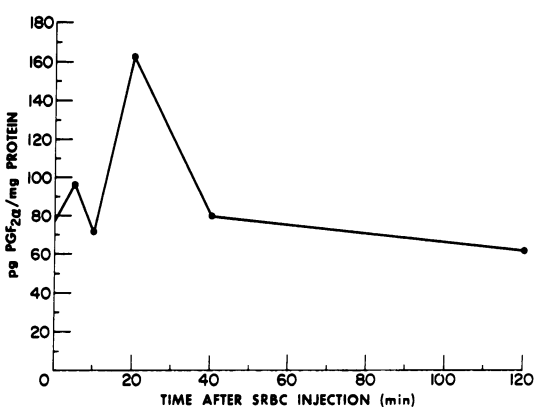

FIG. 2. Changes in thymic $PGF_{2\alpha}$ levels following stimulation *in vivo* by sRBC. C57Bl/6J mice were injected with 2×10^8 sRBC i.v. Control mice receiving saline shown as 0 time values.

Table 3. Splenic $PGF_{2\alpha}$ level in NZB mice

	$PGF_{2\alpha}$, pg/mg of protein*	
	Young	Old
Control	206.8 ± 35.4	505.4 ± 201.4
2 min	725.5 ± 70.7	526.8 ± 148.0
20 min	1050.0 ± 240.7	623.2 ± 177.2

Control mice were injected with 0.2 ml of saline and sacrificed at 2 min; experimental groups were injected with 2×10^8 sRBC, i.v.
* ± Standard error of the mean.

sults are shown in Table 3. One readily apparent difference is that the levels of PG in untreated spleens are 2.5 times as great in the spleens of the old mice as in those of the young mice. The basal levels in both cases are much higher than those observed in normal C57Bl/6J mice (6–10 weeks of age). Whether the same difference exists with "old" normal versus "young" normal mice has not been determined. Upon injection of sRBC, a 5-fold increase in $PGF_{2\alpha}$ levels occurs in the spleens of the young mice, with little change observed in the old mice.

Effects of a Soluble Antigen on Splenic PG Levels. After it had been established that particulate antigens (which are T cell dependent) may induce elevations in splenic PG levels, experiments employing a soluble antigen were carried out. The data presented in Fig. 3 are the results of an experiment in which BGG (50 μg per mouse) was injected intravenously. Splenic PG levels were measured at various times following injection. There is some initial fluctuation between the time of injection and 1 hr, followed by a 7 to 10-fold increase above the control at 2 hr, and then a decline. Beginning at 48 hr, a second 4-fold increase occurs.

Effects of PG Inhibitors on *In Vivo* and *In Vitro* Primary Immune Responses to sRBC. It was clear that sRBC could induce large changes in splenic PG levels and inhibitors of PG synthesis could block these increases. Therefore, experiments were undertaken to discern the effects of PG inhibition on the appearance of antibody-forming cells following exposure to sRBC. In the two experiments shown in Fig. 4A and B, indomethacin or Ro 20-5720 were used to block PG synthesis at the time of exposure to sRBC (1×10^7 sRBC per mouse, i.v.). Both indomethacin (Fig. 4A) and Ro

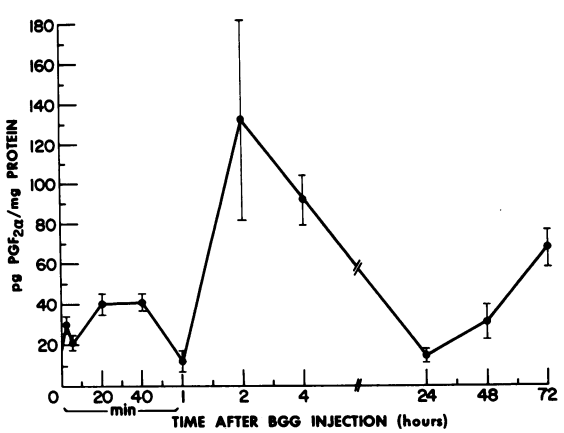

FIG. 3. Changes in splenic $PGF_{2\alpha}$ levels following stimulation *in vivo* by BGG. C57Bl/6J mice were injected with 50 μg of BGG, i.v. Control mice injected with saline shown as 0 time values. Each point represents the mean of values obtained using three mice, ± standard error of the mean.

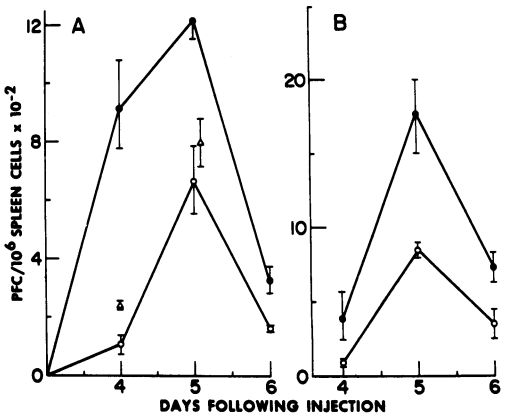

FIG. 4. The effects of inhibition of PG synthesis on the development of cells that directly form antibody against sRBC. (A) C57Bl/6J mice in groups of three were injected with 1×10^7 sRBC, i.v.: ○, sRBC alone; △, 2×10^8 sRBC; ●, sRBC and indomethacin (0.3 mg per mouse, i.p.) injected 24 and 2 hr prior to injection of antigen. (B) C57Bl/6J mice in groups of three were injected with 1×10^7 sRBC i.v.: ○, sRBC alone; ●, sRBC and Ro 20-5720 (0.6 mg per mouse, i.p.) injected at −24 hr, −2 hr and at the same time as antigen injection. Each point represents the mean ± standard error.

20-5720 (Fig. 4B) enhance considerably the appearance of cells that form direct antibody (IgM) against sRBC.

Table 4 depicts the results when three different types of inhibitors of PG synthesis are employed with *in vitro* antibody-forming cell cultures and sRBC is used as the antigen. These results (using two concentrations of sRBC) show that these inhibitors are also quite effective *in vitro* in enhancing the appearance of primary (IgM) antibody-forming cells.

Effect of PG Inhibitors on Mixed Lymphocyte Reactions. All of the experimental data presented above have dealt exclusively with antigen stimulation of the humoral or antibody-forming arm of the immune response. The mixed lymphocyte reaction (MLR), which is an *in vitro* correlate of cellular immunity, was used to test the effects of the PG inhibitors. The results of a typical experiment are shown in Fig. 5. Previous work in this laboratory† and by others (14) had established that a suppressor T cell could be isolated from the spleen based on the property of adherence to glass wool. The population containing suppressor activity weakly adheres to glass wool, whereas the nonadherent population contains the bulk of T cell mitogen-sensitive cells (NAL). Therefore, the effects of the PG inhibitors were tested using whole spleen cells in a two-way MLR; and in addition, a two-way MLR was performed using NAL which are depleted of their suppressor cells. The data in Fig. 5A show that when PG synthesis is inhibited in a whole spleen MLR, a substantial enhancement of DNA synthesis occurs. This is in the absence of any detectable change in cell viability. When the spleen cells are depleted of those adhering to glass wool (containing the mitogen-activated T suppressor cells) (Fig. 5B), a substantial decrease in the effectiveness of PG inhibition is observed so that little enhancement of DNA synthesis occurs. However, it also should be noted that no real enhancement of the NAL MLR occurred over that observed in the whole spleen cultures.

DISCUSSION

The evidence presented here demonstrates that both particulate (sRBC) and soluble antigens (BGG) induce significant

Table 4. Prostaglandin inhibitors enhance the *in vitro* immune response to sRBC

	Day 4, antibody-forming cells/10^6 spleen cells*
sRBC (0.1%)	309 ± 18
sRBC + Ro 20-5720 (500 nM)	489 ± 59†
sRBC + Ro 3-1314 (50 nM)	616 ± 23†
sRBC + indomethacin (50 nM)	613 ± 22†
sRBC (0.01%)	206 ± 9
sRBC + Ro 20-5720 (500 nM)	445 ± 77†
sRBC + indomethacin (50 nM)	443 ± 83†

* Background antibody-forming cells were subtracted. Primary spleen cell cultures were prepared as outlined in *Materials and Methods*, and were assayed 4 days after culture initiation. Values are ± standard error of the mean.

† Probability that value is the same as for sRBC alone, as determined by two-tailed *t* test, $P < 0.01$.

(10 to 80-fold) elevations in splenic prostaglandin ($PGF_{2\alpha}$) levels, and that for BGG these changes occur in a bimodal fashion. Further investigations of sRBC-induced alterations in splenic $PGF_{2\alpha}$ levels have revealed that the early increase is dependent on antigen dose, dependent on the presence of T cells, and restricted largely to spleen cells since only a very limited change occurs in the thymus. Most importantly, two drugs, indomethacin (an irreversible noncompetitive inhibitor) and Ro 20-5720 (a competitive inhibitor of prostaglandin synthetase), when administered to mice prior to the injection of sRBC, almost completely block the early elevation of splenic $PGF_{2\alpha}$. Clearance or phagocytosis of antigen also appears to play a role in the stimulation of splenic prostaglandin levels, since carbon clearance results in an increase in splenic PG (approximately 20–30% that observed using sRBC).

The relevance of the elevation in splenic PG levels to the development of the immune response is indicated by the experiments in which antigen-stimulated prostaglandin synthesis is blocked. These data show that when spleen cells are stimulated either *in vivo* (Fig. 4) or *in vitro* (Table 4) with sRBC, a block of PG synthesis results in an enhancement of the appearance of direct (19 S) antibody-forming cells. That PG synthesis occurs generally following lymphocyte activation is indicated by the mixed lymphocyte reaction, which also shows an enhanced response when PG synthesis is blocked. Furthermore, in a population purified on glass wool (i.e., in the NAL population, MLR) the enhancement of the MLR by PG inhibitors disappears. This suggests the involvement of more than one cell population in the synthesis of PG which may occur during the development of the MLR.

Additional evidence for enhanced responses following PG inhibition has been presented by Webb and Jamieson†. They showed that in phytohemagglutinin-stimulated spleen cell cultures inhibition of PG synthesis enhanced DNA synthesis in whole spleen cell cultures, but not in cultures depleted of T suppressor cell. Also, Ferraris and DeRubertis (10) showed that phytohemagglutinin-stimulated leukocyte cultures yielded 2 to 10-fold increases in PGE levels, beginning 48 hr after culture initiation. They also showed that staphylococcal enterotoxin B stimulated an increase in PG levels in leukocyte cultures, beginning 16 hr after exposure. Since these authors did not measure PG levels at times earlier than 4 hr, they could not detect any of the earlier changes reported here.

The data presented here concerning antigen-stimulated

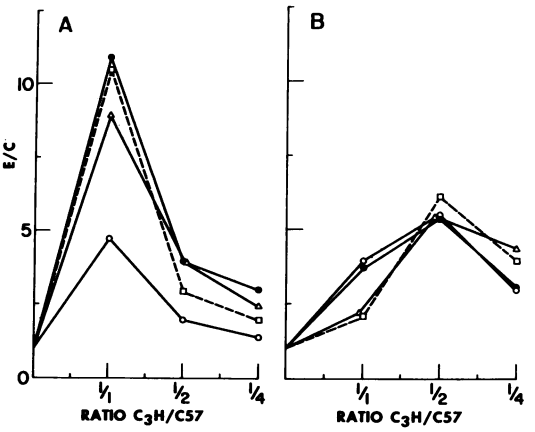

FIG. 5. Effects of the inhibition of PG synthesis on the mixed lymphocyte reaction. (A) C57Bl/6J and C_3H/HeJ mouse spleen cells were cultured in various ratios with or without PG inhibitors. DNA synthesis was measured by [^{3}H]dThd incorporation on day 4 (1 hr pulse). E/C values were computed by Experimental cpm (C57 cpm + C_3H cpm)/Control cpm (C57 and C_3H grown separately). ○, untreated control cultures; ●, cultures incubated with Ro 20-5720, 5×10^{-7} M; △, cultures incubated with Ro 3-1314, 5×10^{-9} M; □, cultures incubated with indomethacin, 5×10^{-9} M. Cell viability at time of assay was >95%; cell recovery was 50–70% of input. (B) DNA synthesis measured in NAL populations isolated from spleens of C57Bl/6J and C_3H/HeJ mice, as outlined in *Materials and Methods*. ○, untreated control cultures; ● cultures incubated with Ro 20-5720, 5×10^{-7} M; △, cultures incubated with Ro 3-1314, 5×10^{-9} M; □, cultures incubated with indomethacin, 5×10^{-9} M. Cell viability at time of assay was >95%; cell recovery was 50–70% of input.

changes obtained using NZB mice are also important. In NZB spleen, the basal levels of $PGF_{2\alpha}$ are elevated compared to the levels found in normal mouse spleen. The NZB mouse spleen is known to be deficient in T cell function (19, 20). It may be speculated that these elevated basal levels are either related to the absence of an endogenous specific cell type, e.g., a T suppressor cell, or are possibly the result of stimulation via autoantigens. Further experiments are necessary to differentiate between these and other possible mechanisms. However, these data do support the concept that PG plays a role in the immune response and the use of such a model may provide much information as to precisely what is the role of PG in the immune system.

Reports from a number of other laboratories have shown that PG, particularly PGE, is effective in modulating immune responses to synthetic antigens (22), as well as affecting the appearance of antibody-forming cells (23). Melmon *et al.* (24) have demonstrated the presence of an apparent receptor on antibody-forming cells, as they were able to remove up to 50% of the spleen cells producing antibody to sRBC by passing them over a column of Sepharose-PGE. Also, in a recent report, Plescia *et al.* (25) have demonstrated that mice made nonresponsive to sRBC by the presence of tumors were restored to immunocompetence by blocking PG synthesis.

Based on the data presented in this report and the work referred to above, we propose that the prostaglandins represent a major hormone class through which regulation and control of immune responses take place. The principal support for this view is derived from the facts that (*1*) athymic mice and NZB mice which lack either T cells or suppressor function show restricted or absent increases in the synthesis of splenic PG in response to sRBC, (*2*) inhibition of PG syn-

thesis enhances all aspects of immunoresponsiveness, including T cell–T cell interactions (MLR, phytohemagglutinin responses) and T cell–bone-marrow-derived (B) cell interactions (*in vivo* and *in vitro* antibody responses), and (3) in those cases where T suppressor cell activity has been detected and removed, such removal largely blocks the enhancement of the response obtained by PG inhibitors.

Concerning the mechanism by which PG exerts its effects, Yamamoto and Webb (26) have recently established that inhibition of splenic PG synthesis also inhibits antigen-stimulated changes in splenic 3′:5′-cAMP and 3′:5′-cGMP levels. The changes in splenic cyclic nucleotide levels in response to sRBC reported by these authors parallel remarkably the changes in splenic $PGF_{2\alpha}$ levels reported here. Since the elevation of cAMP has been found to induce T cell differentiation, a significant proportion of splenic PG may be involved in induction of differentiation in lymphocyte via modulation of cAMP levels (27). Also, changes in cGMP levels have been linked to induction of proliferation (28), increased immune cytolysis (31), and enhanced appearance of antibody-forming cells (32). Thus, alterations in the appropriate PG or its precursors could exert both positive and negative effects on the immune response.

The essential technical assistance of Irene Nowowjieski is gratefully acknowledged.

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