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Similarities in the Suppression of the Immune Response by Interferon and by a Thiol-Oxidizing Agent (40882)[1]

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Abstract. Interferon and oxidized glutathione (GSSG) induced suppression of the C57B1/6 mouse spleen cell *in vitro* plaque-forming cell response. Both suppressions were reversible by 2-mercaptoethanol (2-ME), even when 2-ME was added to cultures up to 48 hr after inhibitors. Other sulfhydryl (SH) reducing agents had a similar effect. *p*-Hydroxymercuribenzene sulfonic acid (PHMB) is strictly a cell surface SH-binding agent, whose suppressive effects were not reversed by delayed addition of 2-ME to cultures. This suggested that 2-ME restored critical free SH groups on the surface of interferon- and GSSG-treated cells, while PHMB-altered SH groups were not restored to the original state. The data are consistent with a model of interferon induction of immunosuppression by binding to SH surface groups of the cell or by functioning as a surface thiol-oxidizing agent. This results in induction of a ribosome-associated protein kinase that blocks initiation of protein synthesis in both interferon- and GSSG-treated cells or cell lysates.

Virus-induced (virus-type) interferon suppresses the *in vitro* antibody response of mouse spleen cells (1–3). We have recently shown that this suppression is blocked by the reducing agent 2-mercaptoethanol (2-ME) (4). The blockage was not due to a direct effect on interferon, since 2-ME was capable of blocking the suppression when added to the cultures up to 48 hr after interferon. This is well beyond the time that interferon has to be present in order to produce the effects that lead to immunosuppression (3).

Several interferon-induced molecular events have recently been described, which may play a role in a variety of interferon-mediated biological activities. One mechanism involves interferon induction of a protein kinase in cells, which in turn catalyzes the phosphorylation of one of the initiation factors (α subunit of eIF-2) required for initiation of protein synthesis (5–8) (see Ref. (9) for a review). eIF-2α is inactive in the phosphorylated state. It has recently been shown in an *in vitro* protein-synthesizing system that oxidized glutathione can also induce the same or similar protein kinase activity that occurs in interferon-treated cells (10–12). These findings coupled with 2-ME blockage of interferon-induced immunosuppression served as the basis for a comparison of the *in vitro* immunosuppressive properties of interferon and oxidized glutathione. If there were a close similarity in the characteristics of the immunosuppressive effects of the two agents, it would suggest that interferon may act as a thiol-oxidizing agent in suppressing the immune response.

Materials and methods. Mice. C57B1/6 female mice were obtained from Jackson Laboratories, Bar Harbor, Maine.

Sheep red blood cells (SRBC). SRBC were obtained from the Colorado Serum Company, Denver, Colorado.

Diluent. Spleen cells at the time of harvesting, SRBC, oxidizing and reducing agents, and interferon were all suspended or diluted in Eagle's minimum essential medium (MEM) containing spinner salts, but no L-glutamine or sodium bicarbonate (3).

Interferon. Mouse L cell interferon was produced in our laboratory as described using NDV as the inducer (13). Portions of the interferon were purified to a specific activity of approximately 10^9 units/mg protein using a combination of absorption (Controlled Pore Glass beads, Electro Nucleonics, Fairfield, N.J.), hydrophobic, and gel filtration chromatography (14–16).

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The purified interferon was used on a selective basis to confirm the results obtained with unpurified interferon.

Reagents. Reagent grade oxidized glutathione (GSSG), L-cysteine, dithiothreitol (DTT), *N*-ethylmaleimide (NEM), iodoacetate, and *p*-hydroxymercuribenzene sulfonic acid (PHMB) were obtained from Sigma Chemicals (St. Louis, Mo.). Reagent grade 2-ME was obtained from Matheson, Coleman, and Bell.

Cultures. Dissociated normal mouse spleen cells were cultured for *in vitro* anti-SRBC plaque-forming cell (PFC) response as described previously (3). PFC responses were determined on Day 5. Details of treatment of cultures with interferon, GSSG, 2-ME, and other reagents are described under Results.

Results. Interferon and GSSG inhibition of the PFC response and the ability of 2-ME to block inhibition. The potent immunosuppressive effects of mouse fibroblast interferon on the PFC response are well established (17). It was of interest to compare the immunosuppressive effects of interferon with those of GSSG, since they cause similar biochemical effects on initiation of protein synthesis (9, 12). The results of such a comparison are presented in Table I. Interferon, as expected, suppressed the PFC response, and this suppression was blocked by 2-ME. GSSG, at concentrations of 1.5×10^{-4} *M* to 1.5×10^{-3} *M*, suppressed the PFC response by 77 to 92%. 2-ME completely blocked the suppression caused by 1.5×10^{-3} *M* GSSG. We previously demonstrated that 2-ME blockage of the immunosuppressive effect of interferon did not involve a direct effect on interferon in solution (4).

It was previously observed that 2-ME was capable of blocking interferon-induced immunosuppression even when added to cultures up to 48 hr after interferon (4). 2-ME similarly blocked GSSG-induced immunosuppression when added to cultures up to 48 hr after GSSG (Fig. 1). The data in Fig. 1 provide further evidence of the similar nature of interferon- and GSSG-induced immunosuppression and suggest that 2-ME does not interact directly with soluble GSSG to block suppression.

Ability of the thiol-reducing agents L-*cysteine and DTT to replace 2-ME in blocking interferon and GSSG immunosuppression.* To ascertain whether 2-ME blocked immunosuppression by possibly acting as a thiol-reducing agent, L-cysteine and DTT were also examined for their ability to block immunosuppression. Both substances were effective in blocking the immunosuppression induced by interferon and GSSG (Table II). DTT was more effective than L-cysteine at blocking interferon- and GSSG-induced suppression, but less effective than 2-ME. The concentrations shown in the table were optimal for

TABLE I. INTERFERON AND GSSG INHIBITION OF THE *IN VITRO* PFC RESPONSE TO SRBC AND THE EFFECT OF 2-ME ON THIS INHIBITION[a]

Interferon (300 units/ml)	GSSG (*M*)	2-ME (5×10^{-5} *M*)	Anti-SRBC PFC/culture (± SD)	Fold inhibition
+	–	–	125 ± 35	31.6
+	–	+	5400 ± 2546	1.1
–	1.5×10^{-3}	–	310 ± 14	12.7
–	1.5×10^{-3}	+	8700 ± 4667	0.7
–	0.5×10^{-3}	–	560 ± 198	7.1
–	1.5×10^{-4}	–	1290 ± 14	3.1
–	0.5×10^{-4}	–	2680 ± 1414	1.5
–	–	–	3950 ± 1344	
–	–	+	5850 ± 1909	

[a] Interferon, GSSG, and 2-ME were added to cultures at the time of SRBC addition. Anti-SRBC PFC responses were determined on Day 5, and are expressed as the mean of duplicate determinations ± SD. The data are from a representative experiment.

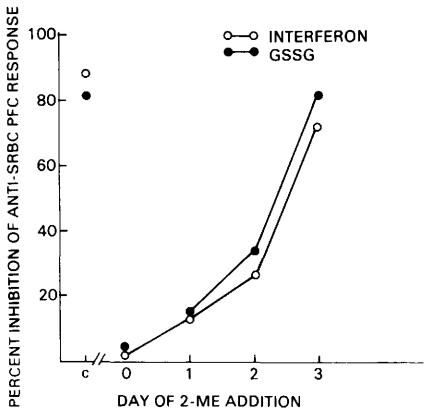

FIG. 1. Effect of time of treatment with 2-ME on interferon and GSSG inhibition of the *in vitro* PFC response to SRBC. Interferon (300 units/ml), GSSG (1.5×10^{-3} *M*), and SRBC were added to cultures at the same time. 2-ME (5×10^{-5} *M*) was added at various days and the anti-SRBC PFC responses were determined on Day 5. The control (C) consisted of interferon or GSSG in the cultures without 2-ME.

blocking suppression as determined by titration. Interferon-induced suppression was more effectively blocked than that of GSSG, but this may be due, in part, to the higher control PFC responses in the GSSG experiments. The patterns of the PFC responses were similar in two repeats of the above experiments. L-cysteine and DTT also blocked suppression when added to cultures up to 24 and 48 hr after interferon and GSSG (data not shown). It is concluded that several thiol-reducing agents are capable of replacing 2-ME in blockage of interferon- and GSSG-induced immunosuppression, and that sulfhydryl interactions are involved.

Effect of sulfhydryl (SH)-reactive agents on the PFC response. GSSG is an oxidative agent for SH groups, and it has previously been reported that other agents capable of reacting with SH groups or oxidizing SH to disulfide inhibited lymphocyte mitogenesis (18). We tested the three SH-binding agents NEM, iodoacetate, and PHMB for their suppressive effect on the PFC response and the reversibility of this suppression by 2-ME (Table III). Conditions of treatment were the same as for GSSG. The concentrations of SH-binding agents used represented equivalent GSSG inhibitory doses as determined by prior titration. All three agents were highly suppressive. 2-ME, 5×10^{-5} *M*, significantly blocked the suppression when added to cultures at the same time as the SH-binding agents. Addition of

TABLE II. RELATIVE ABILITY OF DTT, L-CYSTEINE, AND 2-ME TO BLOCK INTERFERON- AND GSSG-INDUCED IMMUNOSUPPRESSION[a]

Reducing agent (*M*)[b]	Inhibitor[c]	Anti-SRBC PFC/culture (± SD)	Fold inhibition
DTT (10^{-4})	Interferon	5,050 ± 1,061	1.2
DTT (10^{-4})	—	6,050 ± 212	
L-cysteine (10^{-3})	Interferon	3,300 ± 141	1.8
L-cysteine (10^{-3})	—	5,800 ± 424	
2-ME (5×10^{-5})	Interferon	6,700 ± 1,990	1.2
2-ME (5×10^{-5})	—	7,750 ± 1,768	
—	Interferon	125 ± 35	31.6
—	—	3,950 ± 1,344	
DTT (5×10^{-4})	GSSG	3,480 ± 170	2.5
DTT (5×10^{-4})	—	8,800 ± 1,131	
L-cysteine (5×10^{-3})	GSSG	3,760 ± 170	3.5
L-cysteine (5×10^{-3})	—	13,050 ± 4,738	
2-ME (5×10^{-5})	GSSG	12,000 ± 424	1.4
2-ME (5×10^{-5})	—	16,200 ± 1,980	
—	GSSG	230 ± 325	10.3
—	—	2,360 ± 905	

[a] All agents were added to cultures at Day 0.
[b] Concentrations of reducing agents represent optimal doses for blocking suppression.
[c] Interferon, 300 units/ml; GSSG, 1.5×10^{-3} *M*.

TABLE III. INHIBITORY EFFECT OF SULFHYDRYL (SH)-REACTIVE AGENTS ON THE *IN VITRO* PFC RESPONSE TO SRBC AND THE EFFECT OF 2-ME ON THIS INHIBITION[a]

SH-binding agent (M)	2-ME (5×10^{-5} M) (hr)	Anti-SRBC PFC/culture (± SD)	Fold inhibition
NEM (10^{-5})	—	<50	>50
NEM (10^{-5})	0	9,700 ± 2,828	1.2
NEM (10^{-5})	48	150 ± 71	98.0
Iodoacetate (10^{-6})	—	150 ± 71	16.7
Iodoacetate (10^{-6})	0	7,000 ± 1,131	1.7
Iodoacetate (10^{-6})	48	1,500 ± 1,414	9.8
PHMB (10^{-6})	—	50 ± 71	50.0
PHMB (10^{-6})	0	4,340 ± 1,612	2.7
PHMB (10^{-6})	48	30 ± 42	490.0
—	—	2,500 ± 283	
—	0	12,100 ± 283	
—	48	14,700 ± 5,374	

[a] NEM, iodoacetate, and PHMB were added to cultures at the same time as SRBC. 2-ME was added at 0 or 48 hr. Anti-SRBC PFC responses were determined on Day 5, and are expressed as the mean of duplicate determinations ± SD. The data are from a representative experiment. Concentrations of SH-binding agents represent equivalent GSSG inhibitory doses as determined by titration.

2-ME at 48 hr, however, did not reverse the suppression. This is in contrast to GSSG, where addition of 2-ME to cultures at 48 hr significantly blocked or reversed suppression. This may reflect the relative reversibility of the oxidation or alteration of SH groups by GSSG and the other SH agents. The suppressions were probably not due to toxicity, since cell viabilities of treated and untreated cultures as determined by trypan blue dye exclusion were essentially the same (data not shown). PHMB had no effect on SRBC–anti-SRBC hemagglutination. This suggests that the SH-binding agents did not exert their suppressive effects by blockage of the binding of SRBC to specific lymphocyte receptors (data not shown). The best evidence is that PHMB reacts with cell surface SH groups and does not enter the cell (19). Intact surface SH groups, then, may be important for lymphocyte activation.

Discussion. Virus-induced interferon and the thiol-oxidizing agent GSSG are similar in that they have been shown to be capable of inhibiting protein synthesis at the molecular level by the same or similar mechanisms. This involves the induction of protein kinase activity in treated cells or cell lysates, which in turn catalyzes the phosphorylation of one of the initiation factors (α subunit of eIF-2) that is required for protein synthesis. eIF-2 forms a ternary complex with GTP and initiator methionyl transfer RNA in the presence of a stimulating protein. This complex binds to a 40 S ribosomal subunit. Inhibition of translation occurs when eIF-2 is phorphorylated; phosphorylation blocks the interaction of eIF-2 with its stimulating protein and thus prevents formation of the ternary complex (see Ref. (20) for review of protein synthesis).

It was of interest from the biological point of view to determine if interferon and GSSG were similar in their immunosuppressive properties. Interferon suppression of the *in vitro* antibody response was previously shown to be blocked by 2-ME, even when the reducing agent was added to cultures up to 48 hr after interferon. This is well beyond the time that interferon has to be present in order to produce the effects that lead to immunosuppression (4). We have shown here that GSSG also suppressed the antibody response, and that this suppression was blocked or reversed by 2-ME, even when added to cultures as late as 48 hr after GSSG. The ability of 2-ME to block interferon- and GSSG-induced im-

munosuppression was probably due to its thiol-reducing properties, since L-cysteine and DTT had similar effects under the same culture conditions. It was also found that interferon and GSSG were most effective at suppression when added to cultures at the same time as SRBC (data not shown).

NEM, iodoacetate, and PHMB, which are SH-binding agents, also suppressed the antibody response, but 2-ME could not reverse this suppression after these substances were bound to SH groups. This could reflect an irreversible binding of these agents to SH groups that are critical for activation of the lymphocyte. PHMB, because of its strong negative charge, does not enter the cell and binds only to surface SH groups (19). This would suggest that surface SH groups are critical for lymphocyte activation in antibody formation. In related studies it was shown that agents that were capable of binding to or oxidizing free SH groups could inhibit lectin-induced activation of lymphocytes (18). The authors concluded that free SH groups were important during the early phases of induction of lymphocyte activation and suggested that SH interactions were an obligatory step in lymphocyte activation.

The fact that 2-ME could not reverse the suppression induced by NEM, iodoacetate, and PHMB in the absence of killing would suggest that it does not substitute for a blocked cell function. Rather, it would appear that it restores free SH groups in interferon- and GSSG-treated cells, assuming that they affect cell function by a similar mechanism.

It seems plausable to speculate that GSSG added to cultures inhibited lymphocyte function by increasing the intracellular level of GSSG by virtue of its SH-binding and thiol-oxidizing properties. Available data indicate that exogeneous GSSG does not enter the cell (21, 22). It has been shown that agents such as PHMB that alter surface SH groups, inhibit the hexose monophosphate shunt (HMP) pathway; this could result in elevation of intracellular levels of GSSG (19, 23, 24). Elevated levels (as low as 20 μM) of GSSG inhibit initiation of protein synthesis by activation of a protein kinase that phosphorylates a subunit of eIF-2 (12). Interferon inhibits protein synthesis by the same or similar mechanism of protein kinase activation (9). It is tempting, therefore, to suggest that interferon increases cell protein kinase activity and suppresses the immune response by increasing the intracellular concentration of GSSG. Conclusions of this nature, however, will have to await determination of the concentrations of GSSG in interferon-treated cells versus untreated cells. Since the levels of oxidized and reduced glutathione in a cell are primarily maintained at homeostatic levels by generation of NADPH through the HMP pathway, interferon effects on the HMP pathway would also have to be determined. These studies are currently in progress. Certainly, the above model is consistent with our observation that the *in vitro* antibody response is inhibited by ribosome-associated factors from interferon-treated cells that contain protein kinase activity (25).

In preliminary experiments interferon and GSSG also suppressed the PFC response to the T-independent antigen DNP–Ficoll under conditions where T cells were previously removed from the cultures by treatment with anti-Thy-1 and complement. Thus the suppression of the PFC response is not necessarily mediated through T cells.

Finally, direct addition of GSSG to mouse L cells did not block replication of vesicular stomatitis virus. This is consistent with previous data which suggested different mechanisms for the antiviral versus the immunosuppressive properties of interferon (4, 25).

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