Cyclosporin A-treated Guinea Pig Responder Cells Secrete a Genetically Restricted Factor That Suppresses the Mixed Leukocyte Reaction

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ABSTRACT Cyclosporin A (CY A) is a hydrophobic, undecapeptide, fungal metabolite with potent immunosuppressive effects on T lymphocyte-mediated immune responses. Suppressor T lymphocytes generated during a mixed leukocyte reaction (MLR) performed in the presence of CY A, release a factor that suppresses a primary MLR of responder T cells, which is derived from the same strain as the factor producer but lacks specificity for the stimulator cell. These results suggest a finely regulated pathway by which CY A may induce and maintain a permanent state of transplantation tolerance in vivo.

INTRODUCTION

Cyclosporin (CY A),[1] an oligopeptide fungal metabolite, has potent immunosuppressive effects on T cell-mediated immune responses without any remarkable myelotoxicity (1). Administration of CY A markedly prolonged the survival of major histocompatibility complex (MHC) incompatible organ allografts and in some models, grafts were retained by the host even after the administration of CY A was discontinued (2). In vitro, CY A suppressed both T cell-proliferative responses (3) and the induction of cytotoxic cells in the mixed leukocyte reaction (MLR) (4). In our recent studies of the effect of CY A on the MLR in the guinea pig, we have found that in addition to the inhibition of T cell proliferation, CY A led to the induction of a radiosensitive T cell population capable of suppressing the MLR (5). We report here that these CY A-induced T cells, after overnight culture with stimulator cells, but in the absence of CY A, secrete a factor (MLR-SF) with marked suppressive activity in the MLR. As this MLR-SF is restricted in its action by the MHC type of the responding T cell in the MLR, our results demonstrate a finely regulated pathway by which CY A may suppress T cell alloreactivity in vivo.

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[1] *Abbreviations used in this paper*: CY A, cyclosporin; LNC, lymph node cells; MHC, major histocompatibility complex; MLN, mesenteric lymph node; MLR, mixed leukocyte reaction; PEC, peritoneal exudate cells.

METHODS

Alloreactive responder T cells were generated from inbred nonimmune strain 13 or strain 2 mesenteric lymph node (MLN) cells cultured (5×10^6) with nonimmune, irradiated (2,500 R), unfractionated mineral oil-induced peritoneal exudate cells (PEC) (1×10^6) from strain 2 or strain 13 guinea pigs, respectively, for 7 d in 24 well-vessels (Costar, Data Packaging, Cambridge, MA) in 2 ml of culture medium containing RPMI 1640 (Gibco Laboratories, Grand Island Biological Co., Grand Island, NY) supplemented with L-glutamine (300 μg/ml), penicillin (100 U/ml), streptomycin (100 μg/ml), 5-fluorocytosine (1 μg/ml), 2-mercaptoethanol (5×10^{-5} M), and 5% heat-inactivated normal guinea pig serum. Cultures were treated with CY A, 0.1 μg/ml, or with ethanol alone at a 0.01% final concentration. After this period of culture, the recovered cells were washed three times with a large excess of balanced salt solution containing 5% normal guinea pig serum and recultured in complete medium (4×10^6/ml) together with fresh, irradiated allogeneic strain 2 or 13 PEC (1×10^6/ml) for an additional 20 h. These culture supernatants (designated as MLR-SF) were filtered and kept frozen at −20°C until use.

For elimination of T cells, responder alloreactive T cells were divided into two aliquots (6×10^6 cells/ml) of 1 ml each for treatment with antibody and complement. Rabbit

complement (Gibco Laboratories), previously absorbed with guinea pig spleen cells (one spleen per 5 ml of rabbit serum) at 0°C for 1 h, was used at a 1:5 dilution. Either a control mouse ascites, or the ascites 8BE6, containing an anti-guinea pig T cell-specific monoclonal antibody, were added to give a final dilution of 1:500. Cells were incubated for 45 min at 37°C, washed three times, and counted. Control and T cell-depleted lymph node cultures were then each incubated for 20 h in complete medium with irradiated allogeneic PEC as described, and the supernatants were filtered and stored until use.

To test the supernatants for suppressive activity, primary MLR cultures were done as follows. Normal MLN cells from strains 2, 13, $(2 \times 13)F_1$, and BIOB (2×10^6/ml) were mixed with either syngeneic or allogeneic irradiated PEC (2×10^6/ml) in complete culture medium. 100 μl of the cell mixture was added to round-bottom microtiter plates together with either 100 μl of culture medium or 100 μl of the MLR-SF preparation to give the indicated final dilutions. Cultures were kept at 37°C, 5% CO_2 in a humid environment for a total of 5 d. 18 h before harvesting, 1 μCi of [^{3}H]thymidine (6.7 Ci/mmol, New England Nuclear, Boston, MA) was added to each well. Cells were harvested with the aid of a semiautomated harvesting device (Microbiological Associates, Inc., Walkersville, MD) and incorporated radioactivity was counted by liquid scintillation spectroscopy. The results of triplicate cultures are presented as the mean counts per minute. The SE was rarely >10% of the mean and was omitted for simplicity. Percent suppression of the MLR responses was calculated according to the equation, 100 × 1 − counts per minute in the presence of MLR-SF/counts per minute in the presence of medium.

RESULTS

Guinea pig strain 13 lymph node cells (LNC) were cultured for 1 wk with irradiated strain 2 PEC as a source of stimulators in the presence of 0.1 μg/ml of CY A. Control cultures were supplemented with 0.01% ethanol, which was identical to the concentration of ethanol added with CY A to the cultures. After 7 d, the recovered cells were thoroughly washed and recultured for 20 h with strain 2 PEC in the absence of CY A. The resultant cell culture supernatants were then tested for suppressive activity in fresh primary MLR cultures. As shown in Table I, CY A-treated T cells generated a soluble suppressive factor capable of inhibiting the strain 13 anti-strain 2 MLR by 60%; the supernatant collected from the cells not exposed to CY A had no inhibitory effect on the primary MLR. The supernatant from the CY A-treated cultures (Table I, Exp. II) had no effect on the primary strain 2 anti-strain 13 MLR, indicating specificity for the suppressive action of the factor and demonstrating that the inhibition of the primary MLR cultures was not sec-

TABLE I
Production of MLR-SF by CY A-treated MLR Responder Cells

	Source of MLR-SF	Dilution	Responder Strain	Stimulator Strain	[^{3}H]TdR Incorporation	Suppression
	Strain					%
Exp. I	—		13	13	3,595	—
	—		13	2	65,048	—
	13 Anti-2 MLR NO CY A	1:4	13	2	60,300	7
	13 Anti-2 MLR CY A	1:4	13	2	28,930	56
Exp. II	—		13	13	3,562	—
	—		13	2	127,175	—
	13 Anti-2 MLR CY A	1:4	13	2	59,488	55
	13 Anti-2 MLR CY A	1:8	13	2	54,879	59
	13 Anti-2 MLR CY A	1:16	13	2	132,812	0
	—		2	2	1,366	—
	—		2	13	14,860	—
	13 Anti-2 MLR CY A	1:4	2	13	18,353	0
Exp. III	—		2	2	2,086	—
	—		2	13	12,007	—
	2 Anti-2 MLR CY A	1:4	2	13	11,882	1
	2 Anti-13 MLR CY A	1:4	2	13	4,830	60

Strain 13 MLN cells were cultured with strain 2 PEC for 7 d with or without CY A (Exp. I and II), and strain 2 MLN were cultured with strain 2 or 13 PEC for 7 d with CY A (Exp. III). The recovered cells were washed and recultured with fresh strain 2 or 13 PEC for 20 h. The resultant MLR-SF preparation were tested at the indicated dilutions in a primary MLR by using the indicated strain combinations of responder and stimulator cells. MLR cultures were maintained for a total of 5 d, pulsed with [^{3}H]TdR, and the incorporated radioactivity determined.

ondary to carry-over of CY A. We also investigated whether responder LNC that had been cultured with syngeneic stimulator PEC in the presence of CY A could produce a similarly suppressive factor. As shown in Table I, Exp. III, strain 2 LNC primed for 1 wk with syngeneic PEC and CY A, generated no suppressive activity in a strain 2 anti-13 MLR, in marked contrast to strain 2 LNC that were primed with allogeneic strain 13 PEC in the presence of CY A.

As the supernatants were generated from unfractionated LNC, we next determined the cell type responsible for the production of the MLR-SF. Strain 13 LNC were cultured with strain 2 PEC for 7 d in the presence of CY A, washed, and then treated with a monoclonal antibody specific for guinea pig peripheral T lymphocytes (8BE6, J. Chiba and E. M. Shevach, unpublished observations) in the presence of complement or alternatively a control ascites and complement. The antibody-treated LNC were then cultured for 24 h with strain 2 PEC in the absence of CY A. Responder LNC that had been depleted of T cells by treatment with the monoclonal anti-T cell reagent and complement were unable to produce the MLR-SF, thus indicating that T cells were required for its production (Table II).

Our initial experiments with the CY A-induced strain 13 anti-2 MLR-SF demonstrated no effect on the strain 2 anti-13 MLR, thus raising the possibility that the MLR-SF had specificity for the I-region phenotype of the responder T cell population in the MLR, as the MHC of strain 2 and 13 guinea pigs differs only in the I-region. To further localize the cellular element serving as target for the MLR-SF induced by CY A, we made use of a third party strain, the partially inbred BIOB strain. The MHC of BIOB guinea pigs differs in its entire I-region from that of both strain 2 and 13 (6) (B. D. Schwartz, personal communication), and strong MLR responses are generated when BIOB lymphoid cells are used as responders or stimulators with strain 2 or 13 cells. As shown in Table III, a strain 13 anti-2 MLR-SF inhibited both the 13 anti-2 as well as the strain 13 anti-BIOB response, but had no effect on the BIOB anti-2 MLR (Table III, Exp. I). These results suggest that the strain 13 anti-2 MLR-SF is capable of inhibiting the primary MLR response of strain 13 T cells to stimulator cells from any strain; no specificity for stimulator cell Ia antigens was noted. Similar responder T cell specificity was seen with factors derived from strain 2 anti-13 MLR cultures and the strain 2 anti-BIOB combinations. Both factors markedly suppressed the primary MLR responses when strain 2 T cells were the responders, but had little if any effect on the MLR response of the T cells derived from the other strains; again no specificity for the stimulator cell type was apparent. Of particular interest is Exp. IV in Table III where a CY A-derived strain 2 anti-13 MLR-SF had a strong suppressive effect against strain 2 responders proliferating to BIOB stimulators (72% suppression), suppressed less well the response of $(2 \times 13)F_1$ cells to BIOB stimulators (54% suppression), and had no effect on the strain 13 anti-BIOB response.

DISCUSSION

The results presented here demonstrate that primary MLR cultures treated with CY A selectively generate T cells that secrete a genetically restricted factor with potent suppressive effects on the MLR. Although the strains of guinea pigs used in these experiments are not congeneic and we have not yet performed the appropriate backcross analyses to map the action of MLR-SF to the I-region of the guinea pig MHC, the studies with inbred strain 2 and 13 animals whose MHC differ only in the I-region are highly suggestive that the responder T cell population must share the

TABLE II
MLR-SF Is Produced by Guinea Pig T Lymphocytes

Source of MLR-SF	Treatment	Responder Strain	Stimulator Strain	[^{3}H]TdR Incorporation	Suppression
				cpm	*%*
—		13	13	14,662	—
—		13	2	193,508	—
13 Anti-2 MLR CY A	CA + C	13	2	56,994	71
13 Anti-2 MLR CY A	8BE6 + C	13	2	185,884	4

Strain 13 MLN cells were cultured with strain 2 PEC for 7 d in the presence of CY A. The recovered cells were treated with either a control ascites (CA) or with an anti T cell monoclonal antibody (8BE6) and complement. The recovered cells were incubated for 20 h with fresh strain 2 PEC and the resultant MLR-SF was tested in a strain 13 anti-2 primary MLR.

TABLE III
Genetic Restrictions in the Action of CY A-induced MLR-SF

	Source of MLR-SF	Responder Strain	Stimulator Strain	[^{3}A]TdR incorporation		Suppression
				Medium	MLR-SR	
				cpm		%
Exp. I	13 Anti-2 MLR CY A	13	2	363,483	43,120	88
		13	BIOB	174,825	34,709	80
		BIOB	2	161,012	141,114	12
Exp. II	2 Anti-13 MLR CY A	2	13	36,079	12,520	65
		2	BIOB	56,621	16,962	70
		BIOB	13	103,250	98,683	4
Exp. III	2 Anti-BIOB MLR CY A	2	BIOB	28,362	8,098	71
		13	2	89,565	89,305	0
		13	BIOB	38,947	32,509	17
Exp. IV	2 Anti-13 MLR CY A	2	BIOB	28,362	8,007	72
		13	BIOB	38,947	35,144	10
		(2 × 13)F_1	BIOB	32,922	15,180	54

Normal MLN cells from strain 2, 13, (2 × 13)F_1, and BIOB were cultured with either irradiated syngeneic or allogeneic PEC in complete culture medium. The cultures were supplemented with either medium alone or with 100 μl of a CY A-induced MLR-SF preparation to give a final dilution of 1:4 or 1:5. The MLR-SF preparations were obtained by using the indicated strain combinations. Cultures were maintained for a total of 5 d, pulsed with [^{3}H]TdR, harvested, and the incorporated radioactivity was determined. Cultures stimulated with syngeneic PEC gave values that ranged from 449 to 1,945 cpm in this group of experiments.

same I-region haplotype as the factor producer. The genetic restrictions in the action of CY A MLR-SF and its lack of specificity for stimulator antigens make it remarkably similar to the MLR suppressor factors described in the murine MLR in the detailed studies of Rich and Rich (7) and Rich et al. (8). Absorption experiments of the CY A MLR-SF with both normal and activated T cell populations as well as further study of its biochemical properties are currently in progress. It will also be important to determine whether the unique properties of this factor reflect a true lack of stimulator antigen specificity or alternatively that the factor has shed stimulator Ia antigens attached to it in such a manner that only the responder-specific binding site is available for interaction with the T cell.

The precise mechanism by which CY A functions in the induction of this factor is also unclear. We can speculate that although suppressor cells are activated in the normal MLR, their function is obscured because of the strong clonal expansion of alloreactive T cells and the high endogenous production of T cell growth factor. After CY A treatment, which blocks both T cell growth factor production and clonal expansion of alloreactive T cells, there appears to occur a substantial enrichment of suppressor cells allowing for their prompt detection through functional tests (9–11). It seems reasonable to propose that after CY A withdrawal, the activation of these spared suppressor T cells by the stimulator MHC antigens, and the release of suppressor factors specific for alloreactive T cells similar to the one described here, might account for the long standing suppressive effects of the drug. We are presently testing both CY A-induced suppressor T cells as well as CY A MLR-SF for in vivo activity in a guinea pig cardiac allograft model. Although CY A in many respects appears to be an ideal immunosuppressive agent whose use has opened new vistas in transplantation immunology (12, 13), an alternative use for this drug suggested by our data would be the generation of suppressor T cells in vitro. The recent advances (14) in the growth and maintenance of T cell lines as well as the production of antigen-specific T cell hybridomas should allow for the expansion of such CY A-induced T suppressor populations and facilitate the isolation of large quantities of cell-free supernatants which might be capable of inducing and maintaining specific transplantation tolerance in vivo.

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