AN EXPERIMENTAL SYSTEM FOR THE SIMULTANEOUS ESTIMATION OF MITOSTATIC AND LYMPHOTOXIC EFFECTS OF IMMUNOSUPPRESSANTS AND CYTOSTATICS

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(Received for publication 14 September 1970)

Beginning in 1967, our laboratory has published a number of investigations of the nonsyngeneic stem cell-inactivation phenomenon (1–3). It has been shown that, after transplantation of a mixture of allogeneic lymphoid and hemopoietic cells into lethally irradiated hosts, stem elements of the mixture are inactivated by allogenic lymphocytes of the graft. In other words, after transplantation of such a cell mixture exogenous colony-forming units (CFU)[1] give no colonies in recipient spleens. On the basis of this fact we supposed that lymphoid cells of parental genotype, being transplanted into sublethally irradiated F_1 recipients, must inactivate host endogenous CFU, i.e., inhibit endogenous colony formation. The degree of inhibition may serve as a quantitatively accurate value for graft-*versus*-host (GVH) reaction. It is evident that such an experimental system is characterized by the development of two simultaneously occurring processes: multiplication of endogenous CFU and homograft reaction against them by transplanted lymphocytes. Both processes may be quantitatively estimated by counting the number of spleen colonies in animals of corresponding experimental groups. By this technique, it is possible to compare quantitatively two of the most important actions of immunosuppressive agents: their mitostatic effect (CFU inhibition), and their lymphotoxic action; the latter is a true immunosuppressive effect, the abolition of endogenous CFU inactivation.

Materials and Methods

Mice (20–22 g) of CBA, C57BL/6J inbred strains and (CBA × C57BL/6J)F_1 were used in the experiments. Lymph nodes were removed from CBA and C57BL/6J mice and prepared cell suspensions (2) were injected into (CBA × C57BL/6J)F_1 mice in amounts varying from 0.2 to 12 × 10^6 cells. 24 hr before cell transfer, recipients were irradiated with 600 R gamma rays at a dose rate of about 438 R/min. For determination of endogenous CFU number, recipients were sacrificed 9–10 days after irradiation (4).

Immunosuppressants at various dose ranges were injected intraperitoneally into (CBA × C57BL/6J)F_1 mice. The first injection of 6-MP, Imuran, or anti-lymphocyte serum (ALS) was given simultaneously with cell transfer; cyclophosphamide was administered 1–2 hr

[1] *Abbreviations used in this paper:* ALS, anti-lymphocyte serum; CFU, colony-forming units; GVH, graft-*versus*-host.

before transplantation, and hydrocortisone acetate was injected immediately after irradiation (24 hr before cell inoculation). All the preparations were injected daily for 3 days, except cyclophosphamide, which was given for only 2 days. Preparations used were dissolved in saline. In the case of 6-MP and Imuran a minimal amount of 1 N NaOH was added for drug solubilization.

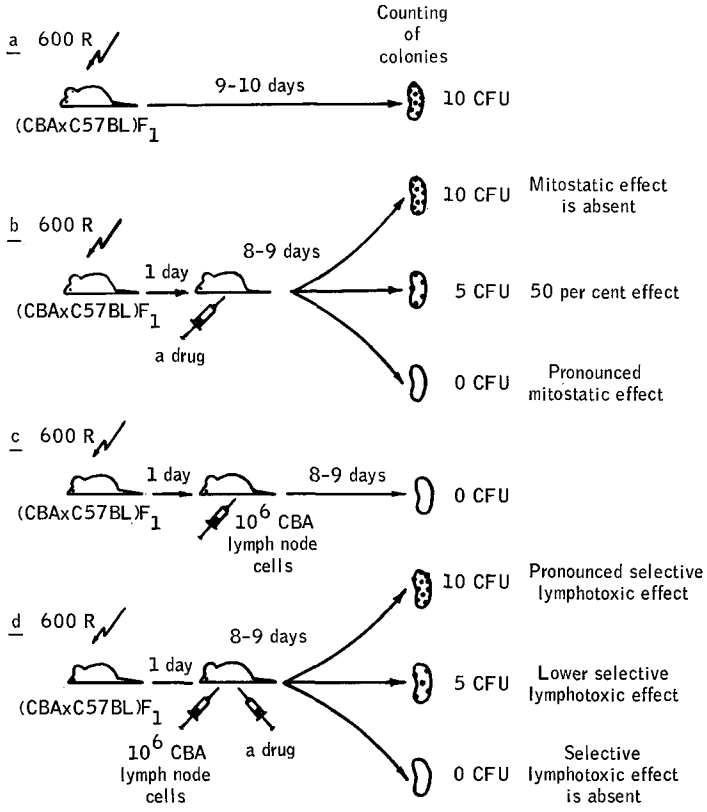

FIG. 1. Experimental schedule for simultaneous quantitative estimation of lymphotoxic and mitostatic effects of immunosuppressants.

In order to obtain ALS,[2] rabbits were immunized with a mixture of mouse spleen and thymus cells. The serum was absorbed with mouse red blood cells and was kept frozen before use. The antiserum obtained caused agglutination of mouse lymphocytes in a dilution of 1:160 and contained 5 mg of protein per 0.1 ml.

A scheme of experiments is shown in Fig. 1. Each experiment includes four major groups: Group *a* (irradiation only) enumerates the endogenous CFU, which developed in the spleens of sublethally irradiated animals at a given radiation dose. Group *b* (irradiation + injection

[2] ALS was prepared by Dr. N. A. Kraskina; we take this opportunity to express to her our grateful acknowledgement.

of preparation) demonstrates the mitostatic effect of a test substance, which is defined as the degree of inhibition of endogenous colony formation. Group *c* (irradiation + injection of parental lymph node cells) demonstrates the activity of transplanted lymphocytes against endogenous CFU, an estimation of GVH reaction. Group *d* (irradiation + transplantation of lymph node cells + injection of preparation) measures simultaneously lymphotoxic and mitostatic action of a test substance.

The last group is the main experimental discriminant. Employing a drug at varying dosages one can obtain different expressions of lymphotoxic and mitostatic properties of the agent. Table I lists possible results.

Variant 1. Colony formation in Group *d* is only slightly suppressed in spite of drug dosage. In this case the drug is highly lymphotoxic, abolishing the homograft reaction of lymphocytes without significant suppression of CFU. This is interpreted to mean that the drug has little mitostatic action. Such a substance would seem to be an excellent immunosuppressive agent.

TABLE I

Variants of Possible Results in the Simultaneous Determinations of Lymphotoxic and Mitostatic Effects of Drugs (a Scheme)

No. of cells grafted into irradiated hosts	Dose of studied preparation, abstract units	No. of endogenous colonies per one spleen (per cent of original)		
		Variant I	Variant II	Variant III
—	—	100	100	100
10^6	—	0	0	0
	1 N	100	0	0
	0.1 N	100	0	50
10^6	0.01 N	100	0	100
	0.001 N	50	0	50
	0.0001 N	0	0	0

Conclusion: Variant I, High selective lymphotoxic effect; mitostatic effect is not expressed. An excellent immunosuppressant. Variant II, selective lymphotoxici action is absent; Mitostatic effect is expressed. Preparation does not qualify as an immunosuppressant. Variant III, selective lymphotoxicity is manifested only at a narrow dose range (0.01 N). Preparation may be used as an immunosuppressant.

Variant II. Endogenous colony formation in Group *d* is absent at any employed dose. Such a drug either has no influence on anything so that lymphocytes completely accomplish the GVH reaction, or the substance possesses a pronounced mitostatic action. In any event such agents do not have a selective action on lymphocytes and should not be used as immunosuppressive agents.

Variant III. It defines drugs with a selective lymphotoxicity only within a narrow dose range.

RESULTS AND DISCUSSION

Following sublethal doses of gamma radiation (600 R) used in our experiments, 10–20 colonies developed in spleens of mice due to endogenous CFU. The data obtained are in accordance with previously reported results (4).

Transplantation of lymphoid elements of parental genotype (CBA or C57BL)

into irradiated F_1 hosts measurably inhibited endogenous colony formation. The degree of endogenous CFU suppression was correlated with the number of injected lymphocytes. Results of experiments are shown in Table II. As seen from the table, 0.2×10^6 lymphoid cells from CBA mice had no influence on the development of endogenous CFU, 0.4×10^6 cells were capable of inactivating about 40% of the endogenous CFU, 0.6×10^6 cells inactivated about 60%, and 1×10^6 cells inhibited multiplication of endogenous CFU almost completely. The dose of lymphoid cells from CBA mice, which caused a 50% inactivation of endogenous CFU was equal to 0.52×10^6 cells. Lymphoid elements from C57BL/6J mice were about one-tenth as active as CBA lymphoid cells.

TABLE II

Inhibition of Endogenous CFU in Sublethally Irradiated (CBA × C57BL/6J)F_1 Mice after Transplantation of Lymphoid Cells from CBA or C57BL/6J Mice

Donor of lymph node cells	No. of grafted cells ($\times 10^6$)	Mean No. ($\bar{x} \pm S\bar{x}$)‡ of endogenous colonies per spleen of sublethally irradiated mice	CFU inhibition	Significance of inhibition
			(%)	(p)
—	—	9.6 ± 0.3 (83)*	—	—
CBA	0.2	10.2 ± 1.1 (16)	Absent	—
CBA	0.4	5.8 ± 0.9 (15)	39.6	0.001
CBA	0.6	3.8 ± 1.6 (5)	60.4	0.001
CBA	1.0	0.3 ± 0.1 (64)	96.9	0.001
CBA	2.0	0.04 ± 0.04 (22)	99.6	0.001
C57BL	2.0	10.4 ± 1.4 (9)	Absent	—
C57BL	4.0	8.1 ± 1.8 (11)	15.6	0.5
C57BL	8.0	1.4 ± 0.4 (12)	85.5	0.001
C57BL	12.0	1.3 ± 0.1 (14)	86.5	0.001

* No. of animals used.

‡ The SE of the mean.

As has been said above, the experimental model allowed us to quantitatively estimate two simultaneously proceeding processes: multiplication of endogenous CFU and reaction of grafted allogeneic lymphocytes against them. In subsequent experiments an attempt was undertaken to employ this model for quantitative characterization of the effects of immunosuppressive agents on these two processes. All these experiments were carried out using transplantation of lymphoid elements from CBA mice. Results of these experiments are presented in Tables III–VII.

Table III characterizes cyclophosphamide action. As can be seen from the table, sublethal irradiation of (CBA × C57BL/6J)F_1 mice resulted in the accumulation in their spleens of 9.5 ± 0.6 endogenous colonies. Injection of the drug at doses of 100 mg/kg was accompanied by a significant mitostatic effect.

The number of endogenous CFU was decreased to 1.9 ± 0.5 (80% inhibition). With decreasing drug concentrations, the mitostatic effect was concomitantly reduced.

At 10 mg/kg and below cyclophosphamide did not influence the development of endogenous CFU. A selective lymphotoxic action of the drug was demonstrated at doses ranging from 2 to 100 mg/kg. But this selective effect was especially manifested at doses of 25 and 50 mg/kg. As was noted above, these doses induced only 16–32% inhibition of colony formation (see "mitostatic effect" column in Table III), for a colony survival of 84–68%.

Approximately the same number of endogenous colonies survived after combined injections of lymphocytes and the drug (79–56%) (see "lymphotoxic

TABLE III

Mitostatic and Lymphotoxic Efficiency of Cyclophosphamide

Dose of preparation, (× 2)*	Mean No. ($\bar{x} \pm S\bar{x}$) of endogenous colonies per spleen in sublethally irradiated mice after injection of the preparation (groups *a* and *b* of Fig. 1)	*CFU* inhibition, (mitostatic effect)	Mean No. ($\bar{x} \pm S\bar{x}$) of endogenous colonies per spleen in sublethally irradiated mice after injection of 10^6 lymphocytes together with preparation (groups *c* and *d* of Fig. 1)	Surviving CFU, (lymphotoxic effect)
(*mg/kg*)		(%)		(%)
—	9.5 ± 0.6 (25)‡	—	0.8 ± 0.5 (20)	8.4
100	1.9 ± 0.5 (14)	80.0	2.5 ± 0.2 (11)	26.3
50	6.5 ± 0.7 (21)	31.6	5.3 ± 0.5 (16)	55.8
25	8.0 ± 1.1 (16)	15.8	7.5 ± 1.5 (13)	78.9
10	9.2 ± 0.2 (16)	3.2	3.5 ± 0.6 (15)	36.8
2	11.2 ± 1.8 (10)	Absent	2.2 ± 0.7 (10)	23.1
0.4	10.3 ± 2.0 (6)	Absent	0.6 ± 0.4 (5)	6.3

* No. of injections of preparation in indicated doses.

‡ No. of animals used.

effect" column). If mice were injected with lymphocytes without the drug only 8.4% of endogenous CFU survived. After combined injections of lymphocytes and appropriate doses of cyclophosphamide, graft-*versus*-host activity of transplanted lymphoid elements was hardly manifested. The drug can therefore be said to have a marked lymphotoxic action. The mitostatic effect of the drug in these concentrations is nearly absent. Larger drug doses (100 mg/kg) cause high mitostatic effect. In smaller doses (2–10 mg/kg), the mitostatic effect of the drug is absent, but the lymphotoxic effect is also poor. At a dose of 0.4 mg/kg, cyclophosphamide had neither mitostatic nor lymphotoxic action.

Hydrocortisone acetate (Table IV) at doses of 25–50 mg/kg caused some mitostatic action on the multiplication of endogenous CFU; 25–37% of colonies were inactivated. At smaller concentrations (0.04–5 mg/kg) a mitostatic effect of hydrocortisone was not registered. A clear selective lymphotoxic action

of the drug was demonstrated at a dose of 5 mg/kg. Mitostatic effect was absent, whereas the drug caused a significant lympotoxic action on some part of the grafted lymphocytes. The percentage of surviving CFU was increased from 10 to 44% in the control and experimental groups respectively.

TABLE IV

Mitostatic and Lymphotoxic Efficiency of Hydrocortisone Acetate

Dose of preparation (× 3)*	Mean No. ($\bar{x} \pm S\bar{x}$) of endogenous colonies per spleen in sublethally irradiated mice after injection of the preparation (groups *a* and *b* of Fig. 1)	CFU inhibition (mitostatic effect)	Mean No. ($\bar{x} \pm S\bar{x}$) of endogenous colonies per spleen in sublethally irradiated mice after injection of 10^6 lymphocytes together with preparation (groups *c* and *d* of Fig. 1)	Surviving CFU (lymphotoxic effect)
(*mg/kg*)		(%)		(%)
—	9.9 ± 0.5 (32)‡	—	1.0 ± 0.4 (27)	10.1
50	7.4 ± 2.0 (7)	25.3	3.3 ± 0.8 (10)	33.3
25	6.2 ± 1.0 (20)	37.4	1.4 ± 0.3 (16)	14.1
5	15.4 ± 2.6 (15)	Absent	4.4 ± 0.88 (20)	44.4
1	14.0 ± 1.6 (12)	Absent	1.1 ± 0.3 (10)	11.1
0.2	10.5 ± 1.3 (6)	Absent	1.5 ± 0.5 (6)	15.1
0.04	13.8 ± 1.5 (6)	Absent	0.7 ± 0.5 (6)	7.0

* No. of injections of preparation in indicated doses.

‡ No. of animals used.

TABLE V

Mitostatic and Lymphotoxic Efficiency of ALS

Dose of preparation, ml per mouse*	Mean No. ($\bar{x} \pm S\bar{x}$) of endogenous colonies per spleen in sublethally irradiated mice after injection of the preparation (groups *a* and *b* of Fig. 1)	CFU inhibition (mitostatic effect)	Mean No. ($\bar{x} \pm S\bar{x}$) of endogenous colonies per spleen in sublethally irradiated mice after injection of 10^6 lymphocytes together with preparation (groups *c* and *d* of Fig. 1)	Surviving CFU (lymphotoxic effect)
		(%)		(%)
—	18.7 ± 2.2 (11)‡	—	0.8 ± 0.4 (12)	4.3
0.4	26.6 ± 1.8 (12)	Absent	13.4 ± 1.9 (12)	71.6

* ALS was injected i.p. 24, 48, and 72 hr after irradiation at doses 0.2, 0.1, and 0.1 ml respectively. Because of the high selective lymphotoxic action further dose titration was not carried out.

‡ No. of animals used.

ALS had a highly selective lymphotoxic action. As can be seen from Table V, ALS failed to show a mitostatic effect at a dose of 0.4 ml. Moreover, stimulation of endogenous CFU multiplication (by a factor of about 1.5) was observed. In addition, ALS possessed pronounced selective activity on lymphoid elements. After allogeneic lymphocyte transfer without injection of the serum,

only 4% of endogenous CFU were recovered, while combined inoculation of lymphocytes together with ALS increased the number of surviving colonies to 71%.

6-MP and Imuran proved to have another character of action. As can be seen

TABLE VI

Mitostatic and Lymphotoxic Efficiency of 6-MP

Dose of preparation (× 3)*	Mean no. ($\bar{x} \pm S\bar{x}$) of endogenous colonies per spleen in sublethally irradiated mice after injection of the preparation (groups *a* and *b* of Fig. 1)	CFU inhibition (mitostatic effect)	Mean no. ($\bar{x} \pm S\bar{x}$) of endogenous colonies per spleen in sublethally irradiated mice after injection of 10⁷ lymphocytes together with preparation (groups *c* and *d* of Fig. 1)	Surviving CFU (lymphotoxic effect)
(*mg/kg*)		(%)		(%)
—	18.7 ± 2.8 (3)‡	—	5.7 ± 2.8 (3)	30.4
60	1.5 ± 1.0 (4)	92.0	0.3 ± 0.3 (3)	1.6
30	2.7 ± 0.8 (4)	85.6	1.7 ± 1.7 (3)	9.1
20	1.0 ± 1.0 (3)	99.5	1.0 ± 0.4 (4)	5.0
10	5.0 ± 1.5 (3)	73.3	0.2 ± 0.2 (4)	1.0
1	7.8 ± 0.2 (5)	58.3	0 (4)	0
0.1	19.5 ± 5.6 (4)	Absent	0 (4)	0

* No. of injections of preparation in indicated doses.

‡ No. of animals used.

TABLE VII

Mitostatic and Lymphotoxic Efficiency of Imuran

Dose of preparation (× 3)*	Mean no. ($\bar{x} \pm S\bar{x}$) of endogenous colonies per spleen in sublethally irradiated mice after injection of the preparation (groups *a* and *b* of Fig. 1)	CFU inhibition (mitostatic effect)	Mean number ($\bar{x} \pm S\bar{x}$) of endogenous colonies per spleen in sublethally irradiated mice after injection of 10^6 lymphocytes together with preparation (groups *c* and *d* of Fig. 1)	Surviving CFU (lymphotoxic effect)
(*mg/kg*)		(%)		(%)
—	20.0 ± 5.5 (3)‡	—	3.67 ± 1.2 (3)	18.3
60	4.3 ± 2.6 (3)	78.5	3.00 ± 1.0 (2)	15.0
30	9.5 ± 5.1 (4)	52.5	2.70 ± 1.7 (3)	13.5
20	21.2 ± 5.4 (4)	Absent	1.5 ± 0.8 (4)	7.5
10	20.0 ± 13.1 (2)	Absent	4 (1)	20.0
1	31.0 ± 3.0 (2)	Absent	2.5 ± 1.5 (2)	12.5

* No. of injections of preparation in indicated doses.

‡ No. of animals used.

from Table VI, 6-MP exerted pronounced mitostatic effects on endogenous CFU without any influence on graft-*versus*-host activity of transplanted lymphoid elements. 20–60 mg/kg of the drug inactivated 86–99% of colonies, 1–10 mg/kg inactivated 58–73%, and only a dose of 0.1 mg/kg had no mitostatic

action on endogenous CFU. No employed dose of 6-MP gave a selective lymphotoxic effect. In all cases, the number of surviving CFU after combined inoculation of 6-MP and lymphocytes failed to exceed the number of colonies surviving after injection of allogeneic lymphocytes without the drug. Imuran had an analogous mode of action (Table VII).

Cyclophosphamide and ALS proved to be drugs with a high dose range of selective lymphotoxic action. Hydrocortisone acetate had a more narrow range of selective lymphotoxic effect. 6-MP and Imuran failed to exert any selective actions on lymphoid elements. They possessed, however, pronounced mitostatic action.

Quantitative estimation of both lymphotoxic and mitostatic effects of drugs and other preparations is important for the evaluation of agents employed to prevent GVH reactions. The search for lymphotropic cytostatics is a great oncological problem. The proposed model may be useful in making rational choices of immunosuppressive agents used not only for suppression of GVH reactions but for organ transplantation (kidney, liver, etc.) as well. However, further investigations of these drugs are required for the final judgement of this question.

SUMMARY

Sublethally (600 R) irradiated (CBA × C57BL)F_1 mice were grafted intravenously with parental lymph node cells in doses ranging from 0.2×10^6 to 12×10^6. The transplantation of these lymphoid cells leads to inactivation of the recipient's endogenous CFU (as measured by the diminution of the number of colonies registered on the 10th day after irradiation). A 50% inactivation was observed when the graft size of the CBA cells was 0.52×10^6. This figure for C57BL cells was 10 times more. This experimental system evaluates two simultaneously developing processes: the multiplication of endogenous CFU and the homograft reaction of transplanted lymphocytes against them. Both processes can be quantitatively estimated simultaneously in the same experiment by the determination of the number of colonies in corresponding experimental groups. Thus it was possible in a single experiment to compare quantitatively the effect of immunosuppressants on two points: (*a*) mitostatic action (suppression of CFU) and (*b*) lymphotoxic action. The latter, a true immunosuppressive effect, represents suppression of GVH activity of lymphoid cells and is demonstrated by abolition of the inhibition of endogenous colony formation. In the present system we have tested 6-MP, ALS, cyclophosphamide, hydrocortisone, and other drugs. The definite mitostatic and lymphotoxic doses of drugs are ascertained. Cyclophosphamide and ALS proved to be drugs with high dose ranges of selective lymphotoxic action. Hydrocortisone acetate had a more narrow range of selective lymphotoxic effect. 6-MP and Imuran (azathioprine) failed to exert any selective action on lymphoid elements. They possessed pronounced mitostatic efficiency, however.

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