

STUDY OF THE CELLS PROLIFERATING IN PARENT VERSUS F₁ HYBRID MIXED LYMPHOCYTE CULTURE*

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The cellular events taking place in mixed lymphocyte cultures (MLC)¹ between cells of parental and F₁ hybrid origin have been usually considered to represent an unidirectional reaction, where only the parental cells are proliferating (1, 2). Using MLC established with mouse spleen cells, it has been observed, however, that F₁ cells can be stimulated by parental cells blocked by irradiation or mitomycin C treatment (3-5). In the present work, parent-hybrid MLC have been performed with cells from different lymphoid organs, and the parental or F₁ origin of dividing cells established by caryotypic analysis. The role of T lymphocytes in the MLC response was examined in cultures of spleen cell suspensions depleted in either parental or hybrid T lymphocytes. Finally, the T or B nature of proliferating cells observed in MLC containing irradiated parental or F₁ cells was studied by immunofluorescence.

Materials and Methods

Mice. CBA/H-T6T6, C57BL/6, BALB/c were purchased from Jackson Laboratories, Bar Harbor, Maine, and bred in our laboratory. F₁ hybrids were obtained by mating C57BL/6 and BALB/c female mice with CBA/H-T6T6 male mice.

Cell Preparation. Cell suspensions were prepared as previously described (6) and spleen cells were submitted to a brief osmotic shock to remove red blood cells (7). Spleen cells depleted in T lymphocytes were prepared by treatment with mouse anti- θ C3H or rabbit anti-mouse T-lymphocyte antigen (MTLA)² and C' as described elsewhere (6). Lymph node cell suspensions were obtained from a pool of peripheral and mesenteric lymph nodes. Cortisone-resistant thymocytes (CR thymocytes) were prepared from mice treated with hydrocortisone acetate according to Blomgren and Andersson (8). Spleen cells depleted in macrophages were prepared according to Chen and Hirsch (9).

Cultures. Each culture was performed in tubes (Falcon Plastics, Div. of BioQuest, Oxnard, Calif., 12 x 75 mm) containing 1 ml of cell suspension in culture medium. Different types of mediums and culture conditions were used. Some cultures were performed in medium RPMI 1640 (Microbio-

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¹ *Abbreviations used in this paper:* BUdR, 5-bromodeoxyuridine; CR thymocytes, cortisone-resistant thymocytes; FCS, fetal calf serum; GVH, graft-vs.-host reaction; MLC, mixed lymphocyte culture; MSLA, mouse specific lymphocyte antigen; MTLA, mouse T-lymphocyte antigen; sIg, surface immunoglobulin; SI, stimulation index; [³H]TdR, tritiated thymidine.

² Rabbit antiserum antimouse thymocyte, rendered specific against mouse T lymphocyte, was previously called anti-MSLA (mouse specific lymphocyte antigen), after Shigeno et al. (10). It seems more appropriate, following Sauser et al. (11) to call this type of antiserum anti-MTLA, which matches better the use of the abbreviation anti-MBLA to describe an antiserum recognizing mouse B lymphocytes (12). The θ -alloantigen is apparently part of the same molecule as the MTLA xeno-antigen (11).

logical Associates, Bethesda, Md.) containing 5% fetal calf serum (FCS, Flow Laboratories Ltd., Irvine, Scotland) or 5% freshly prepared rat serum; these cultures contained $1-2 \times 10^6$ viable cells/ml. Other cultures were performed in the medium described by Click and Peck et al. (13, 7) (hereafter referred to as Click medium), with or without 0.5% mouse serum; these cultures contained $2-4 \times 10^6$ viable cells/ml. In MLC using only viable cells, the ratio of parental to F₁ cells was 1:1. MLC using irradiated cells were performed in Click medium and contained 2.5×10^6 normal cells with 3.5×10^6 irradiated cells. Irradiated cells had received 750 rads in 1-2 min from a Cobalt 80 source. All cultures were kept for 2-5 days at 37°C in a humidified atmosphere (95% air, 5% CO₂).

Determination of [³H]Thymidine Incorporation into DNA and of Stimulation of DNA Synthesis. Cultures were pulsed 6-8 h before harvest with 1 μCi of [³H]thymidine (5 Ci/mmol, Radiochemical Center, Amersham, England), and determination of DNA synthesis was performed as described previously (6). Results presented are the mean cpm of duplicate cultures. In MLC performed with unirradiated cells, the stimulation index (SI) was calculated as the ratio of cpm of [³H]thymidine ([³H]TdR) incorporated into DNA in the cultures containing both parental and F₁ cells to one half the sum of the cpm of [³H]TdR incorporated in the cultures of parental and F₁ cells alone, the number of cells per culture being in all instances equal. When either parental or F₁ cells had been irradiated, the SI was the ratio of the incorporation in semiallogenic cultures to that in syngeneic cultures.

Count of Blasts per Culture. Counts were done according to Häyry et al. (14) on cytocentrifuged Giemsa-stained slides. Blasts were defined as cells with a nuclear diameter larger than 10 μ.

Caryotypic Analysis. Caryotypic analysis was performed on cells pooled from duplicate culture according to the standard technique of Moorhead et al. (15). 40-50 mitoses were scored with a minimum of 30 in less favorable circumstances.

Surface or Intracellular Immunofluorescent Staining. Staining was performed as described previously (16) on cells pooled from several parallel cultures, using a rabbit anti-MTLA antiserum (11) (a kind gift of Dr Sauser, Institute of Biochemistry, Lausanne) for the detection of thymus-derived lymphocytes and a rabbit antimouse Ig for the detection of cells bearing surface Ig (sIg, B lymphocytes) or containing intracellular Ig (iIg). Controls of staining specificity, ruling out the possibility that rabbit Ig might be unspecifically fixed by some blasts present in the cultures, were performed as described elsewhere (16). For the detection of surface Ig, staining with fluorescent antisera was performed on living cells, which were subsequently cytocentrifuged (Shandon Scientific Co, London). For the detection of intracellular Ig, the staining was performed on cytocentrifuged, methanol-fixed cells. In some experiments, surface and intracellular Ig were explored simultaneously, using rabbit antisera bearing a different fluorochrome, as previously shown (16). Preparations were examined with a Zeiss photomicroscope II (Carl Zeiss, Inc., New York). In some cases, the stained slides were also processed for autoradiography, using an Ilford L4 emulsion.

Results

Caryotypic Analysis of the Proliferation of Parental and F₁ Lymphocytes in MLC Performed with Lymphoid Cells of Various Origins

MLC with Spleen Cells. Different culture mediums were used, since it was observed that the SI, and the absolute amount of [³H]thymidine ([³H]TdR) incorporation, varied somewhat depending upon the medium used. In all mediums, however, the SI were usually highest on the 4th day of culture, with a marked decrease on the 5th day. (a) Cultures performed in RPMI medium containing 5% FCS had the lowest SI (3-5), but showed the highest [³H]TdR incorporation, in unstimulated, "background" cultures as well as in MLC. Between the 3rd and 5th day of culture, 20-30% of the cells in mitosis were of F₁ origin. (b) Cultures performed in RPMI medium containing 5% fresh rat serum showed higher SI, mainly due to lower [³H]TdR incorporation in "background" cultures. The percentage of F₁ cells among the dividing cells usually increased from the 3rd to the 5th day of culture, reaching about 40% on day 5 (Table I). (c)

TABLE I
Proliferation of F₁ Lymphocytes in MLC with Parental Cells in Different Culture Conditions and with Cells from Different Origins

Origin of lymphocytes	Culture medium		Day:	3	4	5
Spleen	RPMI + 5% rat serum	SI		6.4 (4-10)*	6.4 (4-8)	3.4 (3-6)
		% of F ₁ mitosis		22% (9-35)*	30% (18-47)	42% (21-57)
		No. of exp. ‡		7	7	6
Spleen	Click + 0.5% mouse serum	SI		6 (3-10)	14 (13-14)	7 (6-7)
		% of F ₁ mitosis		41% (27-64)	51% (50-52)	27% (27-27)
		No. of exp.		3	2	2
Lymph node	RPMI + 5% rat serum	SI		9.6 (6-13)	17 (8-35)	8 (5-13)
		% of F ₁ mitosis		14% (10-17)	21% (13-27)	11% (0-26)
		No. of exp.		2	4	5
CR thymocytes	RPMI + 5% FCS serum	SI		12 (3-36)	15 (8-25)	19 (4-92)
		% of F ₁ mitosis		2% (0-4)	5% (0-12)	1% (0-2)
		No. of exp.		5	6	5
CR thymocytes	Click + 0.5% mouse serum	SI		—	16	—
		% of F ₁ mitosis		—	4%	—
		No. of exp.		—	1	—
Parental spleen + F ₁ CR thymocytes	Click + 0.5% mouse serum	SI		—	4.6 (3-8)	—
		% of F ₁ mitosis		—	5% (0-8)	—
		No. of exp.		—	4	—
Parental spleen + F ₁ CR thymocytes	RPMI + 5% rat serum	SI		—	7 (3-12)	5
		% of F ₁ mitosis		—	42% (18-87)	26%
		No. of exp.		—	3	1

Mean value of MLC between T6T6 parental and (T6 × C57)F₁ lymphocytes.

* Numbers in parentheses refer to the range of values observed.

‡ Number of experiments.

Cultures performed in the medium described by Click et al. (13), containing 0.5% mouse serum, gave the highest SI; about 40–50% of the dividing cells on the 3rd and 4th day of culture were of F₁ origin (Table I). Similar results were observed when this medium was used without mouse serum.

These results clearly establish that not only parental, but also F₁ spleen cells, are stimulated to proliferate during T6T6 vs. (T6 × C57)F₁ spleen cell MLC. Results similar to those presented in Table I were also observed in MLC using the following combination of spleen cells: C57BL/6 vs. (T6 × C57)F₁, T6T6 vs. (T6 × BALB)F₁, and BALB vs. (T6 × BALB)F₁, demonstrating that the proliferation of F₁ cells is not restricted to a peculiar strain combination. In order to appreciate if F₁ proliferation could be modified by sensibilization, F₁ spleen cell donors were repeatedly “immunized” by a weekly injection of 2–3 × 10⁶ parental cells. This was not the case, as cells from these treated donors proliferated to the same extent in MLC with the parental cells as those from untreated mice.

MLC with Lymph Node Cells. The percentage of F₁ cells among the dividing cells on the various days of culture was lower than in the MLC of spleen cells when using the same strain combination and culture conditions (Table I). However, in view of the high SI observed, the stimulation of the F₁ cells is evident.

MLC Between CR Thymocytes. These cultures showed some peculiarities which require comment. First, there was a large variation in the SI. This probably reflected an uneven degree of selection of the responding population of thymocytes by the steroid; indeed, in the conditions used, the recovery of viable thymocytes varied between 3 and 15×10^6 cells/thymus. Second, the total [³H]TdR incorporation and the SI were higher when the cultures contained FCS rather than rat serum; consequently, most cultures were performed in RPMI medium containing 5% FCS (Table I). Nevertheless, good stimulation was also obtained in Click medium containing 0.5% mouse serum (Table I). Third, in contrast to spleen and lymph node culture, the total [³H]TdR incorporation and the SI were most often higher on the 5th day than on the 4th day of culture. The results of caryotype analysis differed strikingly from those of other lymphoid cell cultures, since they showed that, from the 3rd to the 5th day of culture, the cellular proliferation was practically only of parental origin. Addition of 2-mercaptoethanol to the culture (final concentration 1.5×10^{-5} M) did not modify the results.

MLC Between Spleen Cells and CR Thymocytes

MLC WITH PARENTAL CR THYMOCYTES AND F₁ SPLEEN CELLS. A strong stimulation of F₁ cells was observed. A representative example is as follows (3rd day of culture in RPMI medium with 5% rat serum): [³H]TdR incorporation in cultures of parental CR thymocytes alone: 600 cpm; of F₁ spleen cells alone: 1,400 cpm; in MLC: 9,100 cpm (SI:10), with 54% of the mitoses of F₁ origin.

MLC WITH PARENTAL SPLEEN CELLS AND F₁ CR THYMOCYTES. The results varied depending upon the culture conditions. In Click medium with 0.5% mouse serum, essentially only the parental spleen cells proliferated (Table I). In contrast, in RPMI medium with 5% rat serum, proliferation of F₁ CR thymocytes was observed (Table I).

MLC Using Parental or F₁ Spleen Cells Depleted in T Lymphocytes

Parental or F₁ spleen cells were depleted in T lymphocytes by treatment with anti-MTLA or anti- θ antisera + C. The efficiency of the T-cell depletion was ascertained by the failure of cultures of these cell suspensions to be stimulated by PHA (6).

Depletion of parental cells in T lymphocytes abolished the stimulation of DNA synthesis in MLC (Table II). In some experiments, a very weak increase in SI was still found, but since this could also be observed in control cultures containing syngeneic parental cells instead of F₁ cells (Table II and legend), it appears to be related to the cell treatment and not to the MLC. Thus, the increased proliferation of F₁ cells in MLC is entirely dependent on the presence of parental T cells.

In MLC using F₁ cells depleted in T lymphocytes and normal parental cells, the increase in [³H]TdR incorporation was usually significantly higher (50% or more) than in parallel MLC using total F₁ spleen cells, and the proportion of F₁ caryotypes among the mitoses was comparable to that found in the parallel MLC (Table II). Similar results were also obtained in MLC where T-lymphocyte-depleted F₁ cells had been in addition depleted of glass-adherent cells. Thus, it can

TABLE II
Effect of T-Cell Depletion of Parental or F₁ Hybrid Spleen Cells on MLC

Cell treatment	Parental cell culture	F ₁ cell culture	MLC		F ₁ caryotypes
			cpm	SI	
	<i>cpm</i>	<i>cpm</i>			%
0	1,700	2,900	21,000	9	ND
Parental cells with anti θ + C	2,000	2,900	3,500	1.3*	ND
0	600	400	3,600	7	22
F ₁ cells with anti MTLA + C	600	900	5,900	8	28

Results are expressed in cpm of [³H]TdR incorporation in cultures of T6T6 and (T6 × C57)F₁ spleen cells arrested after 3 days (RPMI medium with 5% rat serum). Three experiments of each type were performed with comparable results. Similar results were observed in cultures arrested on day 4 and 5.

* This is an example of the slight increase in SI which was still occasionally observed in MLC using T-depleted parental cells. In this experiment, the control culture, mixing T-depleted parental cells and normal parental cells (instead of F₁ cells) showed an [³H]TdR incorporation of 4,200 (SI:2.1).

be concluded that: (a) The stimulation of parental T cells by F₁ cells does not require the presence of F₁ T cells. (b) The proliferation of F₁ cells in MLC involves B lymphocytes. Whether F₁ T lymphocytes can also be stimulated when present in the culture was further explored.

Immunofluorescence Study of the T or B Nature of the Parental or F₁ Blasts Appearing in MLC

This study was performed in conditions of culture allowing the identification of the parental or F₁ origin of the blasts without requiring chromosomal analysis. It is known that lymphoid cells treated with mitomycin or irradiated before culture do not proliferate but can stimulate parental (4) or even F₁ cells (3–5) in semiallogenic MLC. The following experiments showed that the irradiated cells do not proliferate and are dead by the 4th day of culture: (a) In MLC using parental and F₁ cells which had been both irradiated, there was practically no incorporation of [³H]TdR on the 4th day, and all the cells detectable in the culture were dead as judged by trypan blue exclusion. (b) In MLC in which either parental or F₁ cells had been irradiated, all the mitoses identifiable on the 4th day had the caryotype of the unirradiated cells.

All MLC using either F₁ or parental irradiated cells showed stimulation in DNA synthesis and increase in the absolute number of blasts recovered at the end of the culture, when compared to control cultures performed in the presence of similar numbers of syngeneic irradiated cells (Table III). The stimulation of F₁ spleen cells by irradiated parental cells was somewhat weaker than that of parental spleen cells by irradiated F₁ cells, but was nevertheless significant. The blasts obtained after 4 days of culture were studied by surface immunofluorescence for detection of their T (MTLA) or B (surface Ig) nature, and by intracellular immunofluorescence for the detection of Ig-containing cells. Table III shows the average results observed with parental and F₁ blasts obtained after

TABLE III
Study of the Blasts Present on the 4th day of MLC Using Irradiated Parental or F₁ Cells

MLC	SI	No. of blasts per culture [§]	Percentage of blasts			No. of Ig-containing blasts per culture
			B (sIg ⁺)	T (MTLA ⁺)	Ig-containing	
			%	%	%	
Average results*						
P vs. F ₁ rx	11 (5-18)	—	25 (19-40)	70 (65-75)	12 (9-17)	—
F ₁ vs. P rx	5.1 (2-12)	—	31 (15-36)	47 (35-58)	14 (10-20)	—
Individual exp.‡						
P vs. F ₁ rx	11	810,000	32 (12)	75 (12)	9	72,000
P vs. P rx	—	160,000	ND	ND	6	9,600
F ₁ vs. P rx	8	370,000	22 (7)	58 (22)	14	52,700
F ₁ vs. F ₁ rx	—	90,000	ND	ND	6	5,400

T6T6 and (T6 × C57) F₁ cells were used in cultures performed in Click medium with 0.5% mouse serum. Responding cells were spleen cells in all cases, and irradiated cells were spleen cells in three experiments and lymph node cells in two experiments. Blasts were first defined as the cells labeled after a short pulse of [³H]TdR at the end of the culture, and their study required the combined use of immunofluorescence and autoradiography. Since it was observed that the results were similar to those obtained when only nuclear size (> 10 μ) was used to identify blasts, this latter criterium was used in subsequent experiments.

* Average results of five experiments of each type. Numbers in parentheses are the range of values observed.

‡ In this experiment, the P and F₁ irradiated cells used were obtained from lymph nodes. Number in parentheses refers to the percentage of weakly stained cells: i.e., 32% (12) means that 32% of the blasts were scored as positive, but 12% were only weakly labeled.

§ The SI calculated from the estimated increase in the total number of blasts present in the semiallogenic culture compared to the syngenic culture, was somewhat lower than that given by the increase in DNA synthesis, as is the case in the experiment presented. In the conditions used, the increase of DNA synthesis is considered to be a more accurate estimation of the stimulation.

4 days of culture in five different experiments, and the detailed results of one individual experiment.

The parental blasts present in MLC with irradiated F₁ cells consisted of an average of 70% T blasts, and about 25% B blasts (as judged by their surface Ig). In addition, about 10% of the blasts contained intracellular Ig. There was also some Ig-containing blasts or plasma cells in the control cultures performed with syngenic irradiated cells, but the determination of the absolute number of Ig-containing cells per culture showed that MLC contained at least 5-10 times more Ig-containing cells than the control cultures (Table III). MLC between parental lymph node cells and F₁ irradiated cells was also studied by immunofluorescence. There was only 5-10% of Ig-bearing blasts, and virtually no Ig-containing blasts.

The F₁ spleen blasts present in the MLC stimulated by irradiated parental cells contained about the same percentage of B blasts as MLC of stimulated parental cells, with the same indication that a marked differentiation of stimulated F₁ B cells into Ig-containing cells takes place (Table III, Fig. 1). The percentage of F₁ blasts stained by the anti-MTLA antiserum, was however lower

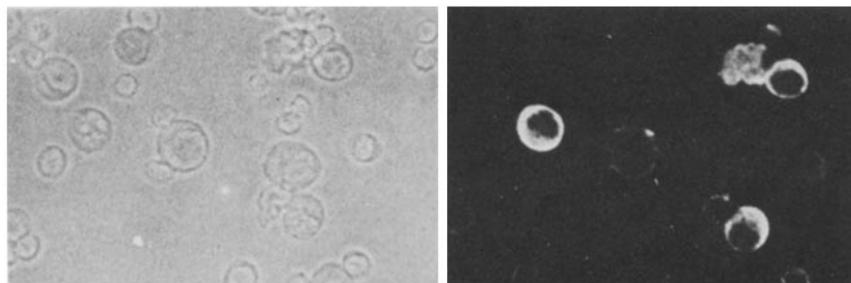


FIG. 1. Cytocentrifuged smear of the cells obtained on the 4th day of an MLC between (T6 \times C57) F₁ spleen cells and irradiated T6T6 spleen cells, and stained by immunofluorescence for the detection of intracellular Ig. Three of the blasts observed by phase-contrast microscopy (left) contain Ig (right). A damaged cell in the right upper corner shows nonspecific fluorescence.

than with parental blast, reaching only about 50%. Furthermore, about half of these cells were only weakly stained, while in the case of parental blasts only 5–10% of the cells identified as T blasts were weakly stained. Finally, all the weakly stained F₁ blasts were not uniformly stained, and there was a gradual decrease of surface fluorescence. Thus, the percentage of F₁ blasts bearing detectable MTLA was in fact more difficult to appraise than with parental blasts, and a sizable proportion of F₁ blasts (about 20%) could be classified neither as T nor as B cells. They did not ingest latex particles, and by phase-contrast microscopy, they did not have the morphology of macrophages. It is possible that these cells were in fact of T origin, but bearing amounts of MTLA antigen too low to be detected by immunofluorescence.

Discussion

The present experiments have allowed, by the use of caryotypic analysis, a direct evaluation of F₁ hybrid cell proliferation in MLC between parental and F₁ lymphoid cells. F₁ cell proliferation, estimated in percentage of F₁ cells among the cells undergoing mitosis on various days of culture, was found to vary markedly depending upon the source of the lymphoid cells used for the MLC: usually strong (sometimes as high as that of the parental cells) in spleen cell MLC, less marked in MLC between lymph node cells, and most often totally absent in MLC between CR thymocytes (Table I). The relationship observed between the extent of the F₁ cell proliferation and the source of cells used for the MLC might explain the discrepant observations reported on the question of F₁ cell stimulation in MLC. In MLC between blood lymphocytes, no F₁ proliferation was detected by caryotypic analysis (1) or study of the blasts' histocompatibility antigens (2), while in spleen cell MLC with mitomycin-treated or irradiated parental cells, stimulation of DNA synthesis in F₁ cells has been reported (3–5), as was also observed in the present experiments. Two additional observations were made concerning the conditions in which the F₁ spleen cells proliferate: (a) The F₁ spleen cell proliferation is entirely dependent upon the presence of parental T cells in the culture (Table II), in confirmation of the results reported by Harrison and Paul (5) and Van Boehmer (17). (b) A marked F₁ spleen cell proliferation is

observed even in the absence of F₁ T cells in the MLC (Table II). Thus, F₁ T lymphocytes are necessary neither for the parental nor for the F₁ cell stimulation. The fact that the F₁ proliferation can consist of B cells, as seen in MLC with T-cell-depleted F₁ cells, is in agreement with the observation that the spleen cells of nude mice, which contain very few T lymphocytes, proliferate in MLC in the presence of irradiated or mitomycin-treated allogenic spleen cells (18, 19). Nevertheless, immunofluorescent analysis of the F₁ cell response (Table III) indicates that F₁ T cells, when present in the culture, are also stimulated.

Two problems will be discussed now: the failure of F₁ cells to proliferate in MLC with CR thymocytes, and the possible mechanisms of F₁ T- and B-cell stimulation in spleen and lymph node MLC. Several possibilities can be ruled out or appear unlikely to explain the lack of F₁ cell stimulation in CR thymocyte MLC. Differences in culture conditions alone did not modify the results. Parental CR thymocytes are competent stimulators for F₁ cells, as demonstrated by the marked proliferation of F₁ spleen cells in MLC between parental CR thymocytes and F₁ spleen cells. Killing of F₁ cells by stimulated CR thymocytes containing a high percentage of cytotoxic cells is not likely since F₁ spleen cells proliferate strongly in the presence of parental CR thymocytes. A more likely possibility is the relative absence among CR thymocyte suspensions of a type of cell which is present in spleen and lymph node suspensions and which is necessary, in addition to parental T lymphocytes, for F₁ T-cell stimulation. It is known that activated macrophages release a product capable of stimulating the proliferation of T lymphocytes (20). Thus, macrophage activation might be necessary for the stimulation of F₁ T lymphocytes, and could take place in MLC through the action of lymphokines released by the stimulated parental T lymphocytes (20, 21). Alternatively, or in addition, it is possible that the F₁ T cells which proliferate in spleen and lymph node MLC consist of a subpopulation of T cells which is not, or poorly, represented among CR thymocytes. When MLC between parental spleen cells and F₁ CR thymocytes were used to explore these possibilities, the results varied depending upon the culture mediums used. F₁ CR thymocytes were not stimulated in one type of medium (which supports a strong proliferation of F₁ T lymphocytes in spleen cells MLC), and stimulated in another medium (Table I). It would appear, therefore, that the lack of F₁ cell proliferation in CR thymocyte MLC results in part from the absence in these cultures of cells which are present in spleen and lymph node MLC, perhaps macrophages, but that, even in the presence of these cells, F₁ CR thymocytes react in MLC somewhat differently from F₁ peripheral T lymphocytes.

The meaning of the F₁ cell proliferation can be best discussed by considering two main hypotheses concerning the nature of the stimulating events: the antigenic stimulation hypotheses, in which the F₁ cells respond to antigenic determinants present on parental cell membranes, and the "regulatory" stimulation hypotheses, in which the F₁ B and T cells respond to the same type of "regulatory" factors and conditions which act *in vivo* on some B and T cells as an indirect consequence of an antigenic stimulation (22). According to the latter hypothesis, the only cells undergoing a specific antigenic stimulation in the culture would be the parental T cells.

It has recently been proposed by Gebhardt et al. (23) that the F₁ spleen cell

proliferation observed in spleen MLC with mitomycin-treated parental cells is a response to parental antigens controlled by genes in or closely linked to the major histocompatibility complex and is analogous to an MLC reaction between allogenic cells. Experiments were reported which were interpreted as indicating a clonal nature of the F_1 response. Thus, when mitomycin-treated cells from both parents were present in the cultures, there was a stronger stimulation of the F_1 than when only one parental population was present. Also, when the cells proliferating in response to the first parent after 2 days of culture were killed with BUdR, addition to the cultures of mitomycin-treated cells of the second parent led to further stimulation of the F_1 cells (23). These observations, however, can also be understood as resulting from a stronger release by mitomycin-treated parental cells of "regulatory" factors stimulatory for the F_1 cells (as discussed below) when cells from both parents are interacting with each other and with the F_1 cells.³ It must be pointed out that if the F_1 cells are stimulated by parental antigens, these antigens would have to be present on parental T cells only, since T-cell depletion of parental cells abolishes the F_1 response, while it is evident that histocompatibility antigens present on F_1 B cells strongly stimulate parental lymphocytes. Furthermore, since F_1 spleen cell populations containing B cells and no T cells proliferate in response to parental cells, one would have to postulate, in the hypothesis that parental cells act only as antigen, that the F_1 response can be thymus-independent. These difficulties might be reconciled by the hypothesis, suggested by the experiments of Ramseier and Lindenmann (24), that the parental antigen to which the F_1 cells respond is the recognition site of the parental T cells for the histocompatibility antigen of the second parent. The F_1 cells cannot, by definition, bear this recognition site, which thus should be antigenic for them. When only F_1 B cells are present in the culture, their proliferation and differentiation in response to this parental antigen might be helped by factors released by the stimulated parental T cells themselves, which would thus not act only as an antigen, but also as the source of an *in vitro* "allogenic effect" (22). It would seem somewhat surprising, however, that this parental antigen could induce a primary immune response generating so many specific antibody-producing cells; furthermore, prior "immunization" of the F_1 mice with parental lymphoid cells, which has been reported to lead to the appearance of F_1 antibodies against the parental T cells recognition sites (24), did not modify the *in vitro* response of the F_1 cells in MLC.

The most direct evidence in favor of the antigenic stimulation hypothesis of the F_1 cells in MLC would be given by the demonstration of an immune specificity of the stimulated F_1 cells towards parental antigens. This has been done neither for

³The experimental scheme of some of these experiments (23) was as follows: F_1 cells were cultured for 2 days with mitomycin-treated cells of parent 1, followed by a "BUdR-suicide" of the proliferating cells and by the addition of mitomycin-treated cells of parent 2, or of parent 1 again as a control. On the 4th day, [³H]TdR incorporation was found to be markedly increased in cultures which had received parent 2 cells, but not in control cultures which had received twice parent 1 cells, suggesting that parent 2 cells had stimulated a second clone. However, it may be noted that in all strain combinations used, addition of parent 2 cells after 48 h led to a much higher increase of [³H]TdR incorporation in cultures stimulated for the first 2 days by parent 1 followed by "BUdR suicide" than in control cultures containing only F_1 cells for the first 2 days. This suggests that the presence of the cells of the two parents in the cultures lead to complex effects.

the T nor for the B F₁ cells stimulated *in vitro*. Some information on this point can be obtained from the observations made *in vivo* in the graft-vs.-host (GVH) reaction, an immunological situation comparable to the MLC between parental and F₁ cells. 1 wk after the injection of parental lymphoid cells into the foot pad of adult F₁ mice or rats there is, in the popliteal lymph node, a marked proliferation of F₁ host cells, consisting mainly of T, B, and Ig-containing cells.⁴ The proliferation of Ig-containing cells appears to represent a polyclonal response, since in the rat it coincides with a strong increase in plaque-forming cells against TNP or sheep red blood cells, although the animals had not been primed to these antigens (25, 26, footnote 4). This would suggest that at least the F₁ B-cell proliferation in the presence of parental T cells is not an immune response to parental antigens, and favors, in our opinion, the hypothesis of a "regulatory" and not of an antigenic stimulation of F₁ cells by parental T cells.

The "regulatory" stimulation hypothesis indeed implies that the signals leading to F₁ cell proliferation are provided by the antigenically stimulated parental T cells present in the culture. Differentiation of F₁ B cells into Ig-producing cells appears in MLC performed in isologous serum, i.e., in the absence of soluble antigens. If the *in vitro* response is indeed polyclonal, as seems to be the case for the *in vivo* response in GVH, this would indicate that the signal for triggering B-lymphocytes differentiation into plasma cells does not necessarily require antigen, a situation for which there are other examples (27). The cells stimulated to differentiate probably belong to a subpopulation of B cells which is rapidly dividing *in vivo*, since MLC established from donors treated before sacrifice with vinblastine, which kills a number of their dividing cells (6), contain much less Ig-producing blasts.⁴ As for the proliferation of F₁ T cells, little is known of the influence of an "allogenic effect" *in vitro* on T cells, although *in vivo* there is evidence that in certain conditions parental T cells injected into an F₁ host can stimulate F₁ T cells (28, 29). All the T blasts found in MLC may not belong to the same subpopulation of T cells, since they were found to vary quite widely in their content of MTLA antigen. Blasts bearing only small amounts of MTLA antigen, as shown by their weak immunofluorescent staining, were especially numerous among F₁ blasts: those also contained blasts on which neither MTLA nor Ig could be detected, and which possibly belong to the same category. Since a "suppressive" effect of T lymphocytes has been described in some immune responses (30), including cell proliferation in MLC (31) and parental cell proliferation in some forms of GVH (29, 32), it can be speculated that at least part of the F₁ T blasts are suppressor or regulator T cells. Some indirect evidence in favor of this interpretation can be found in the observation that depletion of F₁ T cells consistently increased [³H]TdR incorporation in spleen MLC (Table II). Also, there seems to be a correlation between the evolution of the MLC and the amount of F₁ proliferation: spleen cell MLC showed the highest proportion of F₁ cells among dividing cells and a proliferation which decreased markedly between the fourth and the fifth day of culture, while in the CR thymocyte MLC, as well as in the MLC using peripheral blood lymphocytes of rats (1) and mice (2), F₁ cell proliferation was absent and the stimulation of DNA synthesis was still increasing on the fifth day of culture.

⁴Piguet, P. F., H. K. Dewey, and P. Vassalli. Unpublished observation.

Finally, the nature of the parental response in spleen MLC should be discussed. In previous experiments, using MLC performed with spleen cells of radiation chimaeras repopulated with B and T cells bearing a different chromosomal marker, we had observed proliferation of parental B cells (33). By the use of immunofluorescence, the present experiments show, in addition, that a relatively high percentage of the proliferating parental B cells differentiate into Ig-containing cells. However, a notable difference was observed between these two types of spleen MLC: there was a preponderance of parental B mitoses after the first 3 days of culture in MLC performed with the spleen cells of irradiated and reconstituted mice, while in the present experiments, T blasts were predominant at all times of culture. We consider that a difference in the cellular composition of the spleens used for the MLC is the likeliest explanation of these observations. While the spleen cells of animals thymectomized, lethally irradiated and reconstituted in T cells by an injection of thymocytes contain enough T cells to respond in MLC, they probably do not have the same T-cell populations as normal mice, and because of quantitative and/or qualitative differences, they might lack T cells proliferating late in MLC. A difference in the cell populations used might also explain why Andersson et al. (34), studying MLC between allogenic mitomycin-treated cells and artificial mixtures of electrophoretically fractionated populations of B and T cells, failed to observe an appreciable proliferation of B cells. The precursors of Ig-containing blasts, which, as mentioned, seem to belong to a subpopulation of B cells which divide frequently in vivo, might for instance not have been present among the selected cells. All these observations emphasize again the importance of the composition of the cell suspensions placed in culture for determining the events taking place in MLC. If, as seems likely, the parental T cells specifically stimulated by histocompatibility antigens exert, on other parental cell subpopulations, the same kind of influences as discussed to explain the reaction of F_1 cells, the MLC represents a reaction more complex than usually realized. The percentage of specifically reacting cells (35, 36), a problem of great theoretical interest, might thus be difficult to evaluate.

Summary

Caryotypic analysis of the cells dividing in mouse parent-hybrid MLC showed an F_1 hybrid cell proliferation, which varied depending upon the source of lymphoid cells used: strong in spleen MLC (sometimes equal to that of the parental cells), less marked in lymph node cell MLC, and most often absent in MLC between cortisone-resistant (CR) thymocytes. MLC between parental spleen cells and F_1 CR thymocytes showed, however, that in certain conditions of culture, F_1 thymocytes can also proliferate. Using parental or F_1 spleen cells lacking T lymphocytes, it was found that F_1 cell proliferation is entirely dependent upon the presence of parental T cells, but does not require the presence of T lymphocytes among the F_1 cells. Immunofluorescence analysis of the blasts observed in one-way MLC showed that about 70% of the parental blasts were T blasts, and 25% B blasts (containing a high proportion of plasmablasts); among the F_1 blasts, there was also the same percentage of B blasts and plasmablasts, but many of the T blasts bore only small amounts of T-cell antigen (MTLA), and there was also about 20% of unstained blasts,

possibly T blasts bearing MTLA in amounts undetectable by immunofluorescence.

The possibility is discussed that the F₁ responding T cells belong to a subpopulation performing a suppressive function; MLC lacking F₁ T cells showed increased [³H]thymidine incorporation. The proliferation and differentiation of parental and F₁ B cells may result mainly from an unspecific, "polyclonal" triggering.

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