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# Effects of Allogenic and Effector Cells on the Development of Antibody-Producing Cells, *In Vitro* (39099)

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(Introduced by John Doull)

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In 1971, Hirano and Uyeki (1) described a technique for the analysis of cell-mediated immunity *in vitro*. The production of hemolytic plaque-forming cells (PFC) using the *in vitro* technique of Mishell and Dutton (2), served as the indicator for allogeneic and effector cell suppression of PFC production Recently, others (3–6) have also described the effects of allogeneic cells on the development of PFC *in vitro*.

The purpose of this communication is to further describe the properties of allogeneic and effector cells, using the *in vitro* development of PFC as an indicator system.

*Materials and methods.* Female mice of inbred strains C57Bl/6 (H-$2^b$), DBA/2 (H-$2^d$), and $F_1$ hybrids, $BDF_1$ (C57Bl/6 × DBA/2) (H-$2^{bd}$), and $LAF_1$ (C57L/J × A/HeJ) (H-$2^{ab}$) were obtained from Jackson Memorial Laboratories, Bar Harbor, Me. CFW female mice were obtained from Carworth Farms, New York City, N.Y. Mice were used at 8–16 weeks of age.

Fetal calf serum was obtained from Reheis Chemical Co., Kankakee, Ill. Concentrates of minimum essential Eagle's medium (MEM), Hank's balanced salt solution (HBSS), essential amino acids, nonessential amino acids, sodium pyruvate, and guinea pig complement were obtained from Grand Island Biological Co., Grand Island, N.Y. Sheep red blood cells (SRBC) from a single sheep donor were obtained from Mogul Diagnostics, Madison, Wisc. Target spleen cells (TC) from normal mice or from mice primed 4 days previously with $1 \times 10^8$ SRBC were cultured according to the method of Mishell and Dutton (2). The plaque-forming cells (PFC) produced by these cultures are termed target-PFC. The effects of allogeneic spleen cells (AC) or effector spleen cells (EC) on the development of target cell PFC were determined by adding various numbers of allogeneic or effector cells to the target cells during the culture period. Effector cells were generated by injecting either C57Bl/6, $LAF_1$, or CFW mice intraperitoneally with $4 \times 10^7$ $BDF_1$ spleen cells at various times before sacrificing the effector animal. PFC were detected as 19S plaque-forming cells according to a modification of the Jerne technique (7).

Anti-brain-associated-$\theta$ (BA$\theta$) serum was prepared in rabbits and absorbed according to the procedure of Golub (8). This serum killed more than 90% of $BDF_1$ thymocytes at dilutions of $\log_2$ 5. Specific killing of theta-derived spleen cells was done as follows: $4 \times 10^7$ spleen cells were suspended in 1 ml media containing 0.25 ml of anti-BA$\theta$ serum. These cells were incubated for 30 min at 4°C. Thereafter, the cells were spun down, washed once in HBSS, and the pellet was resuspended in 1 ml of 1/8 diluted agar-absorbed guinea pig complement and kept at 4°C for an additional 30 min. Then the cells were spun down and washed twice with HBSS. The viability of these cells was determined by trypan blue exclusion and the remainder of the cells were used in the Mishell–Dutton system (2).

Mice or spleen cells from mice were exposed to 1000 R X irradiation administered at 250 kVp, 0.25 mm Cu as added filtration at a dose rate of 43 R/min. Uv radiation was administered to spleen cells in open tissue culture dishes by a 30 watt G.E. ultraviolet tube for 10 sec at a rate of 2800 erg/sec/cm$^2$.

*Results and discussion.* Our understanding of cellular and molecular events involved in the humoral immune response has been greatly advanced by the culture technique described by Mishell and Dutton (2) for the development of antibody-producing cells *in vitro* from suspensions of mouse spleen cells. The suppression of the development of

TABLE I. EFFECTS OF 1000 ROENTGEN X IRRADIATION ON ALLOGENEIC AND EFFECTOR CELL SUPPRESSION OF TARGET CELL PLAQUE-FORMING CELL PRODUCTION

| Target cells[a] | Allogeneic[b] or, effector cells[c] | 1000 R | PFC/culture[d] (mean and range)[e] |
|---|---|---|---|
| $9 \times 10^6$ n $BDF_1$ | — | — | 9520 (8960–10080) |
| $9 \times 10^6$ n $BDF_1$ | $3 \times 10^6$ AC | — | 470 (425–510) |
| $9 \times 10^6$ n $BDF_1$ | $3 \times 10^6$ AC | Cells[f] | 12,320 (11,360–13,280) |
| $9 \times 10^6$ n $BDF_1$ | $3 \times 10^6$ EC | — | 25 (20–30) |
| $9 \times 10^6$ n $BDF_1$ | $3 \times 10^6$ EC | Cells[f] | 1660 (1090–2230) |
| $9 \times 10^6$ n $BDF_1$ | $3 \times 10^6$ EC | animals[g] | 330 (290–370) |

[a] TC were obtained from spleens of normal $BDF_1$ mice.

[b] Allogeneic cells were obtained from spleens of normal CFW mice.

[c] Effector cells were obtained from spleen cells of CFW mice immunized with $4 \times 10^7$ $BDF_1$ spleen cells 5 days before sacrificing.

[d] $1 \times 10^7$ SRBC were added to each culture. PFC were detected 4 days after planting.

[e] Each number represents the mean of duplicate cultures from two separate groups of mice with the range of the two groups listed in parenthesis.

[f] Cells were suspended in media, placed in tissue culture tube, and exposed to 1000 R X irradiation.

[g] Effector animal was exposed to 1000 R before sacrificing.

plaque-forming cells (PFC) from target spleen cells (target cell-PFC response) by allogeneic or effector cells was used to analyze allogeneic and effector cell interaction *in vitro*. Results of other studies (3–6, 9–11) and our unpublished results show that cells stimulated by mitogens or by allogeneic cell interactions can either enhance or suppress a developing target cell PFC response *in vitro*, depending on the experimental conditions, as well as the AC/TC ratio. In general, when low numbers ($2 \times 10^5$) of allogeneic cells were added to target cells, enhancement of the PFC response occurred, and when large numbers ($2 \times 10^6$) of allogeneic cells were added to target cells, the PFC response was suppressed. Addition of larger numbers ($2 \times 10^6$) to target spleen cells also enhanced the response when suboptimal culture conditions were used (e.g., deficient fetal calf serum). Allogeneic enhancement or suppression of target cell PFC is extremely complex and requires further investigation.

Interpretations of target cell PFC suppression by effector cells may be confused with allogeneic suppression of target cell PFC; therefore, it is of critical necessity that allogeneic suppression can be abolished without interfering with effector cell suppression. Results in Table I illustrate that suppression of target cell PFC production by effector cells was relatively resistant to X irradiation. This was true when either the effector animal or the spleen cells obtained from the effector animal were X irradiated. On the other hand, if allogeneic spleen cells were radiated with 1000 R prior to addition of these cells to target spleen cell cultures, the allogeneic suppression was abolished. We have also found that treatment of cells with uv-radiation for 10 sec at a dose of 2800 erg/sec/cm$^2$ affected allogeneic and effector cell suppression of target cell PFC production in a manner similar to 1000 R X irradiation. Since use of uv radiation was more convenient than X irradiation, uv radiation was used in many of the subsequent experiments to be described.

Whereas, allogeneic cells can either stimulate or suppress the development of PFC from target spleen cells, addition of effector spleen cells always resulted in suppression of target cell PFC production. Also, allogeneic spleen cells only suppressed target cell PFC production when added on Day 0 of culture; conversely, addition of effector cells even on Day 1 of culture resulted in significant suppression of target cell PFC production (Table II). Allogeneic suppression could either be caused by a subpopulation of suppressor T-cells or by the development of cytotoxic effector cells *in vitro*. The differences between allogeneic suppression and effector cell sup-

TABLE II. EFFECTS OF ADDITION OF ALLOGENEIC OR EFFECTOR CELLS, ON DAY 0 OR DAY 1 OF CULTURE, ON TARGET SPLEEN CELL PLAQUE-FORMING CELL PRODUCTION

| Target cells[a] | Allogeneic[b] or effector[c] cells | AC or EC[d] added on | PFC/culture[e] (mean ± SE)[f] |
|---|---|---|---|
| $12 \times 10^6$ n $BDF_1$ | — | — | 9570 ± 650 |
| $12 \times 10^6$ n $BDF_1$ | $3 \times 10^6$ AC | Day 0 | 3050 ± 250 |
| $12 \times 10^6$ n $BDF_1$ | $3 \times 10^6$ AC | Day 1 | 9560 ± 380 |
| $12 \times 10^6$ n $BDF_1$ | $3 \times 10^6$ EC | Day 0 | 90 ± 30 |
| $12 \times 10^6$ n $BDF_1$ | $3 \times 10^6$ EC | Day 1 | 5120 ± 460 |

[a] Target cells were obtained from spleens of normal $BDF_1$ mice.

[b] Allogeneic cells were obtained from spleens of normal CFW mice.

[c] Effector cells were obtained from spleen cells of CFW mice immunized with $4 \times 10^7$ $BDF_1$ spleen cells 5 days before sacrificing.

[d] Day of culture on which AC or EC were added to the TC.

[e] $1 \times 10^7$ SRBC were added to each culture. PFC were detected 4 days after planting.

[f] Each number represents the mean of duplicate cultures from three separate groups of mice ± SEM

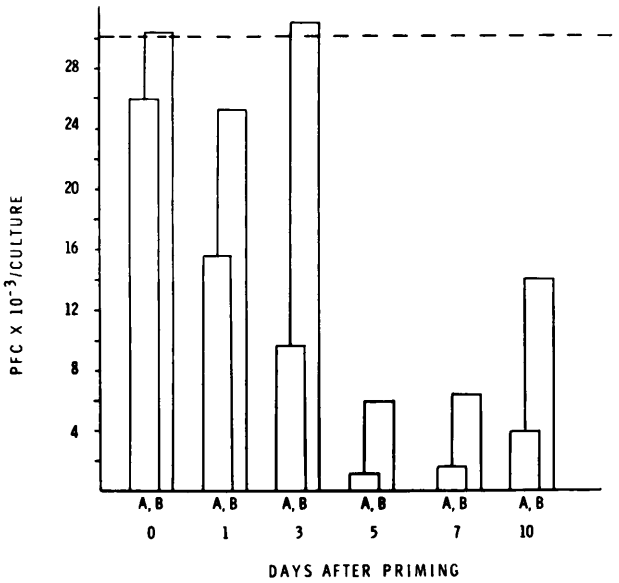


FIG. 1. PFC per culture when $12 \times 10^6$ SRBC primed $BDF_1$ target spleen cells were cultured with $4 \times 10$ $LAF_1$ effector cells obtained from mice at various times after immunization with $4 \times 10^7$ $BDF_1$ spleen cells $1 \times 10^7$ SRBC were added to each culture as an immunogen. PFC were asssessed 4 days after planting. Each bar graph represents the mean value obtained from duplicate cultures from two groups of mice. The dashed line represents the number of PFC obtained when target spleen cells were planted alone. Group A represents cultures in which $4 \times 10^6$ effector cells were added to the target spleen cells. Group B represents cultures in which $4 \times 10^6$ uv-radiated effector cells were added to the target spleen cells (10 sec) of uv light at a dose of 2800 erg/sec/cm$^2$). Day 0 value represents the addition of normal unprimed allogeneic spleen cells.

pression are being evaluated at the present time.

The most reproducible procedure for measuring effector cell suppression of target cell PFC production employed target spleen cells from mice which were primed 4 days previously with $1 \times 10^8$ SRBC, and effector cells that were pretreated with 1000 R X irradiation or with uv radiation for 10 sec at a dose of 2800 erg/sec/cm$^2$. This procedure gave optimal target cell PFC response, thus reflecting true effector cell inhibition.

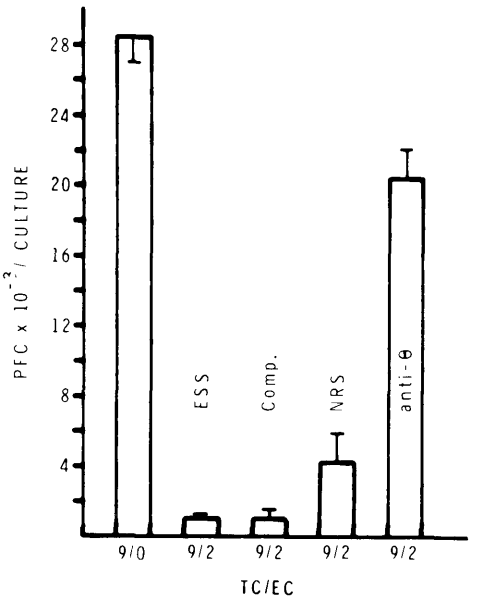


FIG. 2. PFC per culture when $9 \times 10^6$ primed target spleen cells were cultured alone (9/0) or with $2 \times 10^5$ C57Bl/6 effector cells obtained from mice 5 days after immunization with $4 \times 10^7$ $BDF_1$ spleen cells. $1 \times 10^7$ SRBC were added to each culture as an immunogen. PFC were assessed 4 days after planting. Effector cells were pretreated with anti-BA$\theta$ serum plus complement, normal rabbit serum plus complement, complement alone, or media as described in Materials and Methods. Each bar graph represents the mean (± SE) values obtained from duplicate cultures from three groups of mice.

Results in Fig. 1 (bar graph A) illustrate that even 1-day-primed effector cells showed greater suppression of target cell PFC production than did unprimed allogeneic cells (Day 1 vs Day 0). This early (Day 1 and Day 3) effector cell suppression was sensitive to radiation (bar graphs A vs B). Maximum effector cell activity occurred 5–7 days after immunization, and at this time, effector cell activity was relatively resistant to radiation. Ten days after immunization, effector cell suppression began to decline and became more sensitive to radiation. These studies may indicate that allogeneic suppression and effector cell suppression are functions of the same population of cells with a radiation-sensitive amplification step required before T-cell factors reach suppressor or cytotoxic levels. If this amplification step was induced *in vivo*, but not completed by 1 or 3 days after immunization, these effector cells would still be sensitive to radiation. This could also explain why allogeneic suppression only occurred when allogeneic cells were added on Day 0 of culture (Table II), since with the addition of allogeneic cells, this amplification step must take place *in vitro*. Also, this amplification step must occur early in order that suppressor cells exert their activity during the suppressor-sensitive period of PFC production.

Addition of $2 \times 10^6$ effector spleen cells (obtained from C57Bl/6 mice immunized 5 days previously with $4 \times 10^7$ $BDF_1$ spleen cells) suppressed PFC production of $9 \times 10^6$ SRBC primed $BDF_1$ target spleen cells to 4% of control (Fig. 2). Pretreatment of these effector cells with anti-BA$\theta$ serum plus complement abolished this effector cell suppression, whereas pretreatment with normal rabbit serum (NRS) plus complement, or complement alone had relatively marginal effects on effector cell suppression. Viability of effector spleen cells was reduced to 52% after pretreatment with anti-BA$\theta$ serum, whereas the viability of the various control treated groups ranged from 70–88%. Radiation resistance and anti-BA$\theta$ serum-sensitivity of effector cell suppression of target cell PFC production suggest that this assay system may detect activity of mature effector cells. Since our target cells are a proliferating population of cells, this assay system has a "built-in" amplification system. That is, for every PFC precursor cell that is destroyed or suppressed on Day 0 of culture, a large reduction in the number of PFC occurs on Day 4 of culture. Due to this amplification process, this is an extremely sensitive assay system for detecting effector cell activity; activity was seen with EC/TC ratios as low as 1/9.

The specificity of allogeneic and effector cell suppression of target cell PFC production with this system is illustrated in Figs. 3 and 4, respectively. Fig. 3 shows the degree of allogeneic suppression which occurred when increasing numbers of normal C57Bl/6 spleen cells were added to $9 \times 10^6$ target spleen cells obtained from different strains of SRBC-primed mice. Suppression of PFC production by C57Bl/6 ($H\text{-}2^b$) spleen cells occurred against all allogeneic target spleen

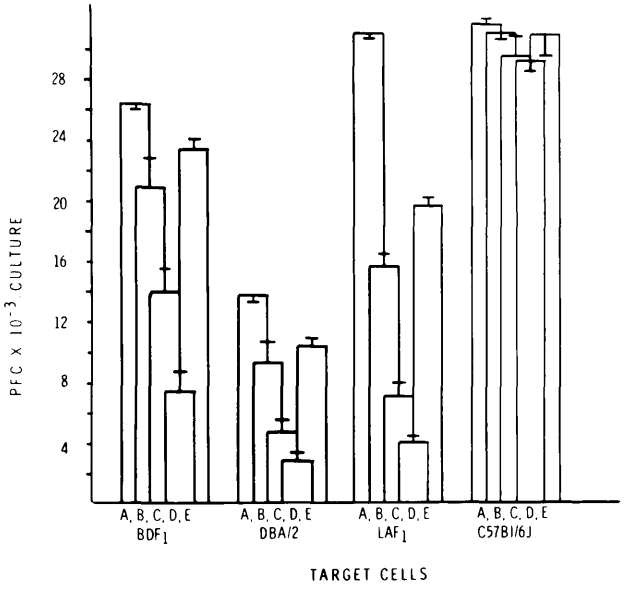


FIG. 3. PFC per culture when $9 \times 10^6$ SRBC primed target spleen cells from $BDF_1$, DBA/2, $LAF_1$, or C57Bl/6 mice were cultured with increasing numbers of allogeneic spleen cells obtained from normal C57Bl/6 mice. $1 \times 10^7$ SRBC were added to each culture as the immunogen. PFC were assessed 4 days after planting. Each bar graph represents the mean (± SE) values obtained from duplicate cultures from three groups of mice. Bar graph A represents PFC obtained from $9 \times 10^6$ target cells alone; bar graph B represents PFC obtained from $9 \times 10^6$ target cells plus $1 \times 10^6$ allogeneic cells; bar graph C represents PFC obtained from $9 \times 10^6$ target cells plus $2 \times 10^6$ allogeneic cells; bar graph D represents PFC obtained from $9 \times 10^6$ target cells plus $3 \times 10^6$ allogeneic cells; and bar graph E represents PFC obtained from $9 \times 10^6$ target cells plus $3 \times 10^6$ uv-radiated allogeneic cells (10 sec of uv light at a dose of 2800 erg/sec/cm²).

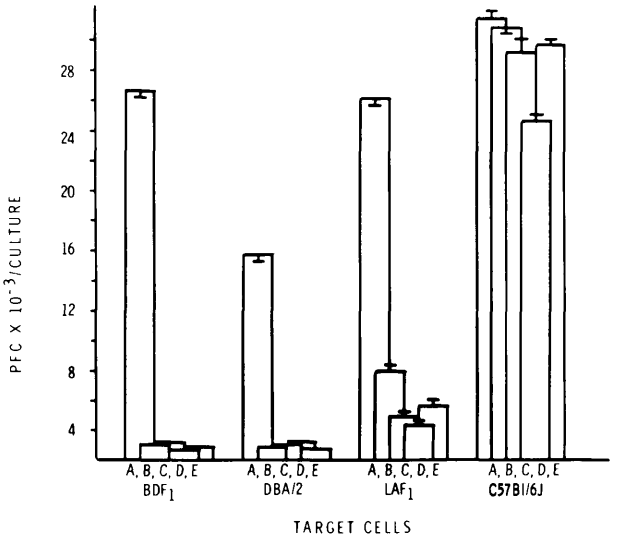


FIG. 4. PFC per culture when $9 \times 10^6$ SRBC primed target spleen cells from $BDF_1$, DBA/2, $LAF_1$, or C57Bl/6 mice were cultured with increasing numbers of effector spleen cells obtained from C57Bl/6 mice immunized 5 days previously with $4 \times 10^7$ $BDF_1$ spleen cells. $1 \times 10^7$ SRBC were added to each culture as the immunogen. PFC were assessed 4 days after planting. Each bar graph represents the mean (± SE) values obtained from duplicate cultures from three groups of mice. Bar graph A represents PFC obtained from $9 \times 10^6$ target cells alone; bar graph B represents PFC obtained from $9 \times 10^6$ target cells plus $1 \times 10^6$ effector cells; bar graph C represents PFC obtained from $9 \times 10^6$ target cells plus $2 \times 10^6$ effector cells; bar graph D represents PFC obtained from $9 \times 10^6$ target cells plus $3 \times 10^6$ effector cells; and bar graph E represents PFC obtained from $9 \times 10^6$ target cells plus $3 \times 10^6$ uv-radiated effector cells (10 sec of uv light at a dose of 2800 erg/sec/cm²).

cells ($BDF_1$, H-$2^{bd}$; DBA/2, H-$2^d$; $LAF_1$, H-$2^{ab}$), but not against syngeneic target spleen cells (C57Bl/6, H-$2^b$). The degree of suppression increased as the dose of allogeneic cells was increased from $1 \times 10^6$ to $3 \times 10^6$ cells/culture. The greatest degree of suppression occurred when $LAF_1$ spleen cells were utilized as target cells for PFC production. If the allogeneic cells were pretreated with uv light, allogeneic suppression was abolished. Results in Fig. 4 illustrate the specificity of effector cells generated. In these experiments anti-H-$2^d$ effector cells were generated by immunizing parental C57Bl/6 (H-$2^b$) mice with $BDF_1$ (H-$2^{bd}$) hybrid offspring spleen cells. As expected, these effector cells greatly reduced the number of PFC produced by $BDF_1$ (H-$2^{bd}$) and DBA/2 (H-$2^d$) target spleen cells with essentially no reduction in C57Bl/6 (H-$2^b$). Anti-H-$2^d$ effector cell suppression of $LAF_1$ (H-$2^{ab}$) target cell PFC production was also expected, since H-$2^{ab}$ is really H-$2^{kdb}$ (12). This effector cell activity was resistant to uv radiation.

We suggest that suppression of *in vitro* PFC production from SRBC primed target spleen cells by radiated effector cells represents a sensitive assay system for detecting effector cell activity. We have used this system to concomitantly analyze humoral immunity and cell-mediated immunity from drug-treated mice (unpublished data). Analyses of allogeneic suppression or enhancement of target cell PFC production are also being pursued.

*Summary.* Effects of allogeneic and effector spleen cells on the development of anti-SRBC plaque-forming cells from target spleen cells *in vitro* were studied. Addition of allogeneic cells resulted in various degrees of suppression of target cell PFC production. This allogeneic suppression could be abolished by pretreating the cells with either uv or X irradiation. Addition of effector cells to target spleen cell cultures markedly suppressed PFC production. Effector cell suppression of PFC production was anti-BA$\theta$ serum-sensitive and radiation-resistant.

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