Use of a Human Plaque-Forming Cell Assay to Study Peripheral Blood Bursa-Equivalent Cell Activation and Excessive Suppressor Cell Activity in Humoral Immunodeficiency

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ABSTRACT A plaque assay that detects human mononuclear blood cells producing immunoglobulin (Ig)M antibody to sheep erythrocytes was investigated for its usefulness in studying B-cell activation and regulation in 24 patients with humoral immunodeficiency. Cells from 3 of 15 patients with common variable agammaglobulinemia produced some plaques (range 40–160/10^6 cells; normal range 80–1240/10^6), but those from the other 12, from all 7 with x-linked agammaglobulinemia and from the 2 with x-linked immunodeficiency with hyper-IgM failed to produce any detectable plaques. In co-cultures of patient and normal cells a very good correlation was seen between results of the plaque assay and an IgM biosynthesis assay in detecting excessive suppressor cell activity. Cells from 7 of 15 common variable agammaglobulinemics, from 3 of 7 x-linked agammaglobulinemics, and from both patients with hyper-IgM caused significant suppression of IgM biosynthesis and(or) plaque formation by normal cells. The observations in the last two groups and discordance for excess suppressor activity in identical twins with common variable agammaglobulinemia suggest that the activity develops secondarily to whatever their primary defects may be. Culturing non-T cells from common variable agammaglobulinemics exhibiting excessive suppressor cell activity with normal T cells resulted in plaque formation in four of five patients so studied; in all five the suppressor activity was found in the T-cell population. The availability of a plaque assay for the study of blood cells from immunodeficient patients provides a new probe to examine the cellular nature of such defects.

INTRODUCTION

Laboratory investigations of patients with primary immunodeficiency have provided useful insights into the functioning of the human immune system. Key among these is the recent appreciation that such disorders may not all be caused by an intrinsic inability of stem cells to fully differentiate into mature lymphoid cells (1–4). In some forms of immunoglobulin deficiency, apparently mature bursa-equivalent (B) lymphocytes are present in normal numbers (2). In a number of such patients, excessive suppressive activity of non-bursa-equivalent cell populations has been demonstrated, raising the question of an etiological role for such cells in the pathogenesis of these conditions (3). Among these are common variable agammaglobulinemia (4–6), hypogammaglobulinemia with benign thymoma (5, 7), some cases of selective Immunoglobulin (Ig)A deficiency (8), and the hypogammaglobulinemia accompanying multiple myeloma (9, 10). There is also some preliminary evidence to suggest that certain patients with x-linked agammaglobulinemia may have excessive suppressor cell activity (11, 12).

The development of assays with cultured human peripheral blood mononuclear cells stimulated with pokeweed mitogen has made it possible to begin to probe into some of the regulatory mechanisms involved in the activation of B lymphocytes for immunoglobulin synthesis and secretion (4, 13, 14). One such recently described procedure, the plaque-forming cell assay (15), has considerable potential for evaluating not only the

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intrinsic capacity of B lymphocytes to differentiate into antibody-forming cells, but also the roles of both suppressor and helper cells in the induction of antibody synthesis. In the studies reported here, we used this assay to study B-cell activation in patients with well-defined primary immunodeficiency disorders and compared its usefulness with that of the immunoglobulin biosynthesis assay described by Waldmann et al. (4). Our results show that the plaque-forming cell assay is capable of detecting excessive suppressor cell activity in these patients and that, for this purpose, it is comparable to the immunoglobulin biosynthetic assay. In addition, it has the added advantage of allowing measurement of specific IgM antibody-forming capacity in these patients.

METHODS

Patient population. This consisted of 24 patients with well-defined primary immunodeficiency diseases (16), including 15 with common variable agammaglobulinemia (ages 20 mo to 24 yr), 7 with x-linked agammaglobulinemia (ages 18 mo to 15 yr), and 2 with x-linked immunodeficiency with hyper IgM (ages 7 and 10 yr). The patients with common variable agammaglobulinemia all had low or undetectable serum immunoglobulins but normal percentages of circulating IgM- and IgD-bearing B lymphocytes. Those with x-linked agammaglobulinemia also had low or undetectable serum immunoglobulins but lacked IgM- or IgD-bearing circulating B lymphocytes. Both patients with hyper-IgM had no detectable serum IgG or IgA by single radial diffusion, markedly elevated serum IgM concentrations, and normal percentages of IgM- and IgD-bearing B lymphocytes. The clinical, immunological, and lymphocyte functional characteristics of many of these patients have been reported (17); similar criteria were used to define those not described previously. All patients were on some form of humoral replacement therapy at the time of these studies. Eight patients received intramuscular immune serum globulin, eight patients received an investigational intravenous immune serum globulin preparation, and eight patients received plasma therapy. Normal controls ranged in age from 20 mo to 45 yr. Written informed consent was obtained from all donors or their parents, and the studies were conducted with the approval of the Duke University Committee on Human Investigation.

Isolation of mononuclear cells. Lymphocytes and monocytes were separated from fresh heparinized venous blood by a modification of the Ficoll-Hypaque (Ficoll, Pharmacia Fine Chemicals Inc., Piscataway, N.J., Hypaque, Winthrop Laboratories, Sterling Drug Co., New York) bouyant density gradient method of Boyum (18), as previously described (19).

Fractionation of mononuclear cells. In some assays Ficoll-Hypaque-isolated, peripheral blood mononuclear cells were separated into thymus-derived (T) lymphocyte enriched, T-cell depleted (non-T), or monocyte-depleted (nonadherent) populations. Equal volumes of mononuclear cells ($2-4 \times 10^6$/ml) and a 2% suspension of neuraminidase-treated sheep erythrocytes (SRBC)[1] (20) were mixed in 15- or 50-ml centrifuge tubes, centrifuged for 5 min at 200 *g* at room temperature, and incubated for 1 h at room temperature. The cell mixture was then resuspended, under-layered with Ficoll-Hypaque, and centrifuged for 30 min at 400 *g* at room temperature. The nonrosetted cells were removed from the interface and the SRBC in the pellet were lysed with Tris-NH_4Cl. In some experiments, further enrichment of T cells was accomplished by allowing them to reform rosettes with SRBC and again centrifuging them on Ficoll-Hypaque. Monocytes were depleted by dispensing 3-ml aliquots of a $4-6 \times 10^6$/ml unfractionated mononuclear cell suspension in RPMI that contained 20% fetal calf serum into 60 × 15-mm plastic dishes (No. 3002 Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, Calif.) and incubating them for 90 min at 37°C in a humidified atmosphere. The nonadherent cells were removed by gentle washing, then 3-ml aliquots of RPMI 1640 that contained 20% fetal calf serum and 15 mM lidocaine hydrochloride were added to the dishes and incubated at room temperature for 60 min (21). The released adherent cells were then washed and tested for viability by trypan blue exclusion.

Cell culture and plaque-forming cell assay. Mononuclear cells were cultured according to the method of Fauci and Pratt (15) in RPMI 1640 medium supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin sulfate, 2 mM L-glutamine, 2.5 mM Hepes buffer, 1% Trypticase-soy broth (BBL Microbiology Systems, Becton, Dickinson & Co., Cockeysville, Md.), and 10% pooled AB serum. The latter was first heat-activated at 56°C for 30 min and then absorbed two to three times with SRBC to remove anti-SRBC antibody, which is known to cause artifactual plaques (22). Cells were cultured at concentrations ranging from 0.5 to 2.5×10^6/ml in a volume of 1 ml in 12 × 75 mm polystyrene tubes (No. 2003 Falcon Labware). When cells or subpopulations of cells were co-cultured, the maximum concentration never exceeded 2×10^6 cells/ml and the volume was usually held at 1 ml. Cultures were either left unstimulated or stimulated with varying concentrations of pokeweed mitogen ranging from 1:30 to 1:5,000 as the final dilution and incubated for varying periods of time (usually 5 or 6 d) at 37°C in a 5% CO_2 humidified atmosphere. In some experiments cultures were stimulated with varying doses of the following agents instead of pokeweed mitogen: phytohemagglutinin-*P* (Difco Laboratories, Detroit, Mich.), concanavalin A (Sigma Chemical Co., St. Louis, Mo.), tetanus toxoid (Massachusetts Public Health Biologic Laboratories, Brookline, Mass.), pneumococcal polysaccharide type III (a gift from Dr. Philip Baker), or TNP-Ficoll (a gift from Dr. David Scott). At the end of the culture period, cells were centrifuged and washed twice in RPMI 1640 medium as described above but without AB serum. The cells were resuspended in 0.5–1 ml of this medium for plaquing.

The plaque assay was performed according to the method of Dosch and Gelfand (23). Wells of flat-bottomed Microtest II plastic trays (No. 3040 Falcon Labware) were each filled with 50 μl of poly-L-lysine (Sigma Chemical Co., mol wt 60,000) at a concentration of 100 μg/ml of distilled H_2O, incubated at 37°C for 30 min, then washed with phosphate-buffered saline. To each well were then added 100 μl of a 1–3% suspension of washed erythrocytes. In most cases the indicator cells were SRBC but in some experiments the indicators were either SRBC or goat erythrocytes with various antigens coupled to their surfaces by a chromic chloride method (24). The trays were then either reincubated for 30–60 min at 37°C or centrifuged for 5 min at 500 rpm, after which excess SRBC were rinsed off with phosphate-buffered saline. To each well were then added 25–50 μl of the cultured mononuclear cell suspensions, containing from 5×10^3 to 5×10^4 cells, and an equal volume of a 1:16 dilution of SRBC-absorbed guinea pig complement. All assays were done in triplicate or quadruplicate. After a 45- to 60-min incubation at 37°C, the trays were removed and the numbers of hemolytic plaques enumerated under low power magnification, with a dissecting microscope. In each

[1] *Abbreviation used in this paper*: SRBC, sheep erythrocytes.

tray, selected plaques were examined under high power magnification with an inverted microscope to determine whether mononuclear cells were present in the lytic areas. In most instances lytic plaques contained from 1 to 12 mononuclear cells.

The number of plaques observed were expressed finally as the number per 10^6 cultured mononuclear cells. The numbers reported in this paper represent the maximum number obtained with any dose of pokeweed mitogen. The expected number of plaques in the co-cultures was determined by the following calculation: expected number of plaque-forming cells per 10^6 cells in co-culture of cells from donors A + B = (number of plaque-forming cells per 10^6 A + plaque-forming cells per 10^6 B)/2. The percentage of the expected number of plaque-forming cells represented by those actually observed was determined by dividing the expected number of plaque-forming cells per 10^6 cells in co-culture into the actual number of plaque-forming cells per 10^6 cells in co-culture and multiplying by 100 to obtain the percentage of predicted.

Treatment of plaque-forming cells. In some experiments monospecific rabbit antisera to human μ- and δ-immunoglobulin heavy chains (prepared in this laboratory) were added at dilutions ranging from 1:10 to 1:400 to wells of Microtest II trays at the time of plaquing. In other studies, puromycin (Sigma Chemical Co.) at a concentration of 10 μg/ml was added to selected cultures 24 h before harvesting for the plaque assay; this dose of puromycin did not decrease cell viability.

IgM biosynthesis assay. Immunoglobulin biosynthesis studies were carried out according to the method of Waldmann et al. (4). Mononuclear cells were removed from the Ficoll-Hypaque interface and thrice washed in 300 mosM, 7.2 pH phosphate-buffered saline that contained 2% fetal calf serum to ensure removal of all Ficoll-Hypaque. They were then washed thrice more in the same phosphate-buffered saline but with 20% fetal calf serum. Cultures were conducted in the same medium as described above for the plaque assay, except that Trypticase-soy broth was omitted and 10% fetal calf serum was used in lieu of human AB serum. Cultures were set up in duplicate in glass tubes at a concentration of 2×10^6 cells/ml and were incubated for 7 d at 37°C in a 5% CO_2 humidified atmosphere. They were either left unstimulated or stimulated by adding 3 or 10 μl/ml of pokeweed mitogen. Co-cultures were performed in the same manner, except that 2×10^6 mononuclear cells from each individual were added in 1-ml vol to give a final volume of 2 ml. Harvesting of cultures was performed by centrifuging the tubes at 400 *g* for 10 min. The supernates were withdrawn and stored at −20°C until assayed for IgM. The latter was done by the double antibody radioimmunoassay as described by Gleich et al. (25). ^{125}I-labeled Waldenström's IgM was purified by gel exclusion chromatography on Sephadex G-200 (Pharmacia Fine Chemicals Inc.); monospecific rabbit anti-human μ:chain and donkey anti-rabbit IgG were prepared in this laboratory by immunizing the animals with the purified proteins in complete Freund's adjuvant. Results of the co-cultures were expressed as the total amount of IgM produced per culture. The predicted amount was calculated by summing the amounts of immunoglobulin synthesized in cultures of each individual alone. The percent suppression was calculated by dividing the observed amount of immunoglobulin synthesized in the co-culture by the predicted amount, multiplying by 100, then subtracting from 100.

Lymphocyte proliferation assay. The methods employed in this laboratory for measuring DNA synthesis after lymphocyte stimulation have been described in detail (17). Briefly, for pokeweed mitogen stimulation, 1×10^5 mononuclear cells were incubated in 0.2-ml cultures in flat-bottomed microplates (Linbro Chemical Co., Hamden, Conn.). RPMI-1640 medium supplemented with 20% pooled human AB serum, 50 μm/ml penicillin, 50 μg/ml streptomycin sulfate, 2.5 mM Hepes buffer, and 2 mM L-glutamine was used. In each experiment several doses of pokeweed mitogen were used for stimulation, and responses were evaluated on 2 successive d (days 5 and 6 of culture). 6 h before the cultures were harvested they were pulsed with 1 μCi [*methyl*-^{3}H]thymidine (2 Ci/mmol, sp act, New England Nuclear, Boston, Mass.). The cells were harvested with a 24-well semi-automated harvester (Otto Hiller, Madison, Wis.) onto glass fiber filter paper (H. Reeve Angel & Co., Inc., Clifton, N. J.). The paper disks were then counted in an LS-230 liquid scintillation spectrometer.

RESULTS

Culture conditions. Experiments were undertaken to determine optimal conditions for the plaque assay. As has been reported, the peak plaque-forming cell response after pokeweed mitogen stimulation occurred on days 5 and 6 (15). Few plaques were detected before day 4 and the number had fallen off sharply by day 7.

The optimal concentration of mononuclear cells placed in culture was also determined. The number of plaque-forming cells/10^6 mononuclear cells was quite similar over a range of concentrations from 0.5 to 2.5 $\times$ 10^6 cells/culture. Most studies in the present report were, therefore, done with either 1 or 2×10^6 cells/ml of media. The minimum number of plaque-forming cells we could observe at these cell concentrations was 20–40/10^6 mononuclear cells.

Numerous attempts were made to induce plaque-forming cells by culturing with various antigens and mitogens other than pokeweed. The results of these experiments are presented in Table I. Cells incubated in the presence of different doses of phytohemagglutinin and concanavalin A were plaqued against sheep erythrocytes on different days. Cells cultured in the presence of different doses of specific antigens were plaqued

TABLE I
Plaque-Forming Cell Response of Peripheral Blood Mononuclear Cells after Incubation with Various Stimuli

Stimulus	Range	Number of experiments
	PFC/10^6	
Background	<20–40	30
PWM	194–2250	25
PHA	<20	3
Con A	<20	4
Tetanus toxoid	<20–60	8
SRBC	<20	5
SIII	<20	3
TNP-Ficoll	<20	2

Abbreviations used in this table: Con A, concanavalin A; PFC, plaque-forming cells; PHA, phytohemagglutinin; PWM, pokeweed mitogen; SIII, stimulus type III (pneumococcal polysaccharide).

against the appropriate antigen coupled to an indicator cell over the course of several days. In our hands only pokeweed mitogen stimulation led to reproducible plaque formation and, thus far, only against sheep erythrocyte antigens.

Plaque-forming cell assay. Plaque formation was dependent upon the presence of viable cells and complement. Plaques could be blocked by the anti-μ specific antiserum but not by anti-δ at any concentration. Plaques were also dependent on *de novo* protein synthesis. Puromycin, added at doses known to inhibit protein synthesis 24 h before plaquing, inhibited plaque formation without increasing the percentage of nonviable cells.

The number of plaque-forming cells per 10^6 cells in culture was determined with mononuclear cells from 18 normal individuals. There was great variability in the ability of cells from individual donors to form plaques (range 80–1440 plaque-forming cells/10^6). When the same individual's cells were evaluated on different days, however, they tended to give responses that were of the same order of magnitude. The dose of pokeweed mitogen inducing the maximal number of plaques also varied from individual to individual but ranged from 1:40 to a 1:1,000 final concentration in culture. The mean numbers of plaque-forming cells found in studies of peripheral blood cells from normal individuals and from patients with various immunodeficiencies are shown in Fig. 1. 3 of the 15 patients with common variable agammaglobulinemia produced some plaques (range 40–160 plaque-forming cells/10^6). 12 of the patients with common variable agammaglobulinemia and all 7 of those with x-linked agammaglobulinemia failed to produce plaque-forming cells. The two patients with x-linked immunodeficiency with hyper-IgM also failed to produce plaques.

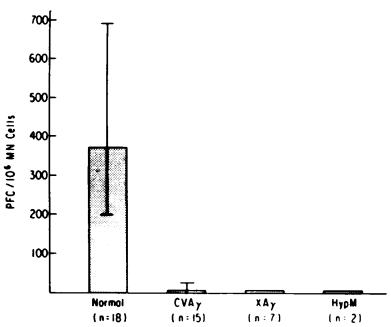

FIGURE 1 Comparison of mean number of plaque-forming cells (PFC) per 10^6 cells produced by mononuclear blood cells from 18 normal controls, from 15 patients with common variable agammaglobulinemia (CVAγ), from 7 with x-linked agammaglobulinemia (XAγ), and from 2 with x-linked immunodeficiency with hyper-IgM (HypM). Vertical lines represent ±1 SD of the geometric mean. n, number of individuals in each group.

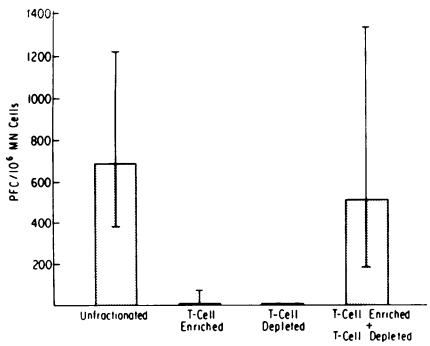

FIGURE 2 Comparison of the capacity of various normal mononuclear cell subpopulations to generate plaque-forming cells (PFC). The results depicted represent the geometric mean number of plaques produced per 10^6 cells ±1 SD when results from five separate experiments were pooled.

Cellular requirements for plaque formation. Experiments were designed to determine what cell subpopulations were necessary for plaque formation. T cell-enriched and T cell-depleted populations were obtained by SRBC rosetting and cultured in the presence of pokeweed, as described above. T cell-enriched populations contained <2% cells that stained with fluoresceinated anti-IgM or IgD. T cell-depleted populations generally showed >64% cells bearing surface IgM or IgD. Monocyte-depleted populations were obtained as described in Methods. Fig. 2 depicts the results of these experiments. Whereas T cell-depleted populations lacked plaque-forming cells, the T cell-enriched populations did produce some plaque-forming cells in three of seven experiments. When T cell-enriched populations were added to T cell-depleted populations, the plaque-forming cell response was restored toward normal.

Detection of suppression in co-cultures. To determine whether the plaque-forming cell assay could detect the presence of excessive suppressor cell activity, co-cultures were made of mononuclear cells from normal individuals and patients with immunodeficiencies. Tables II and III present the results of studies in which mononuclear cells from various normal individuals were co-cultured with those from other normals, from 15 patients with common variable agammaglobulinemia, from 7 with x-linked agammaglobulinemia and from 2 with hyper-IgM. In 11 co-cultures of cells from different normal individuals, the observed number of plaques was 62–150% of the predicted number, with the mean being 86% of predicted (Table II).

Co-cultures of cells from normal controls and from 8 of 15 common variable agammaglobulinemic patients gave plaque-forming cell assay results that were on the average 106% of predicted (range 84–180%) (Table III). When these results were compared with those obtained in the IgM biosynthetic assay, it was found that patients

TABLE II
Results of Plaque-Forming Cell Co-culture Studies with Normal Subjects' Mononuclear Cells

Co-culture	Expected	Observed	Percentage expected
	Plaque-forming cells/10^6 cells		
1	480	300	63
2	340	360	106
3	650	400	62
4	280	200	71
5	260	200	77
6	280	200	71
7	320	320	100
8	360	280	78
9	380	340	85
10	330	280	88
11	120	180	150
Means	321	269	86

who failed to suppress in the plaque-forming cell assay also did not suppress the synthesis of IgM in vitro. In contrast, mononuclear cells from the other seven common variable agammaglobulinemic patients suppressed plaque formation by 75–100% when co-cultured with normal individuals. Among those common variable agammaglobulinemic patients who did suppress in the plaque-forming cell assay, all but patient 15 also showed at least 50% suppression in the IgM biosynthesis assay. The latter patient is a female with very low serum IgG and IgA but a normal IgM concentration. It is of interest that cells from patient 9 were capable of suppressing plaque formation in co-culture with control cells whereas those from his identical twin brother, patient No. 3, did not. Studies on these brothers were done on three occasions and the results were consistent in each instance.

There was also evidence for excessive suppressor activity in some of the patients with x-linked agammaglobulinemia (Table III). None of these patients' cells made plaque-forming cells when cultured individually (Fig. 1). In co-cultures of mononuclear cells from normal individuals with cells from patients 1, 2, and 3 there was 100, 100, and 68% suppression, respectively, in the plaque-forming cell assay. Patient 1 expired before IgM biosynthetic studies could be performed, but such studies in patients 2 and 3 showed 87 and 53% suppression, respectively. In contrast, cells from patients 4–7 gave little suggestion of excessive suppressor activity as measured either by the plaque-forming cell assay or IgM biosynthesis. In fact, co-cultures of normal cells with those from patient 5 yielded a much higher number of plaque-forming cells than expected in experiments with three different normal controls. The three patients showing evidence of increased suppressor cell activity are of particular interest because they were each afflicted with persistent enterovirus infections of the central nervous system (26), whereas the patients who did not suppress did not have such infections.

Co-cultures of mononuclear cells from normal controls and from the two patients with hyper-IgM showed no plaque formation at all (Table III), indicating complete suppression. Cells from each of these patients also suppressed IgM biosynthesis by ≅70%.

Cellular requirements for suppression. A series of studies was conducted to determine if the cellular suppression noted was the property of a specific subpopulation of lymphocytes. We studied SRBC rosetting (T) cells and mononuclear cells depleted of SRBC rosetting cells (non-T) from five of the seven patients with common variable agammaglobulinemia whose unfractionated mononuclear cells had demonstrated excessive suppressor activity. The results of a typical study are shown in Fig. 3. Patient T cells, but not patient non-T cells, were capable of suppressing almost completely the expected number of plaque-forming cells when co-cultured with normal unfractionated mononuclear cells. Non-T cells from the patient and T cells from a normal individual were incapable of producing plaques when cultured alone. In contrast, non-T (i.e., B cell-enriched) cells from this patient were able to produce a large number of plaques when cultured with purified normal T cells. These results were typical of results from three more of the five patients studied. The fifth patient's T-depleted cells did not produce plaques when cultured with normal T cells. Cells from two patients whose unfractionated cells did not suppress in co-cultures with normal cells were also separated and evaluated in a similar manner. In both instances their non-T cells failed to produce plaques when co-cultured with normal T cells. Finally, purified monocytes from two patients with common variable agammaglobulinemia whose unfractionated mononuclear cells suppressed normal plaque-forming cell capacity were isolated as described. Addition of the nonadherent fraction to normal unfractionated mononuclear cells gave the expected suppressions. Adherent cells recovered and washed were added in varying numbers to unfractionated normal cells. These adherent cells failed to cause any suppression. The results of one of these experiments are shown in Fig. 4.

DNA synthetic response to pokeweed mitogen. A possible explanation for the failure of cells from the immunodeficiency patients to produce plaques is that cells from such patients are incapable of giving a blastogenic response to pokeweed mitogen. Table IV indicates that cells from all patients studied did undergo significant DNA synthesis in the presence of pokeweed mitogen.

DISCUSSION

The studies detailed here confirm recent reports from other laboratories that a proportion of human peripheral blood mononuclear cells can be induced to become hemolytic plaque-forming cells in vitro (14, 15, 23, 28, 29). For years assays of this type have proven of immense value in studying the in vitro antibody-forming capacity of single lymphoid cells from experimental animals (27). Their availability now for the study of human primary immunodeficiency disorders provides a new probe to examine the cellular nature of such defects, because it allows in vitro measurement of specific IgM antibody-forming capacity. Other currently available methods for studying human B-cell activation in vitro, those measuring immunoglobulin synthesis and secretion (4, 30) or allowing enumeration of cells with intracytoplasmic immunoglobulin (5, 7, 13), are limited in their usefulness, as they do not measure specific antibody production. In the present

TABLE III

Results of Co-Culture Studies with Normal and Immunodeficient Patients' Mononuclear Cells as Measured by the Plaque-Forming Cell and Immunoglobulin Biosynthesis Assays

Co-Culture	PFC			IgM biosynthesis		
	Expected	Observed	Percentage expected	Expected	Observed	Percentage expected
	plaque-forming cells/10^6 cells			*ng IgM/culture supernate*		
CVAγ + N1 (group 1)						
1	180	120	67	956	1,120	127
2	140	120	86	1,948	2,670	137
3	220	200	91	1,828	916	50
4	500	440	88	—	—	—
5	640	540	84	—	—	—
6	200	360	180	2,610	1,600	61
7	200	280	140	2,610	1,770	68
8	360	420	117	890	974	109
Means	267	271	107	1,658	1,400	92
CVAγ + N1 (group 2)						
9	220	<20	<9	1,824	522	29
10	260	<40	15	1,366	616	45
11	220	<20	<9	2,660	0	0
12	180	<20	<11	—	—	—
13	400	<20	<5	3,230	1,160	35
14	400	<20	<5	3,230	148	4
15	140	<20	<14	1,160	685	59
Means	243	<22	<9.7	2,077	183	29
XAγ + N1						
1	480	<20	<4	—	—	—
2	120	<20	<17	1,500	196	13
3	380	120	32	1,160	550	47
4	160	240	150	600	429	71
5	280	440	157	326	930	280
6	150	160	107	—	—	—
7	320	280	88	—	—	—
Means	241	112	<79	763	455	103
HypM						
1	180	<20	<11	1,605	508	30
2	960	<20	<2	850	262	31

Abbreviations used in this table: CVAγ, Common variable agammaglobulinemia; HypM, immunodeficiency with hyper-IgM; N1, normal control; XAγ, x-linked agammaglobulinemia.

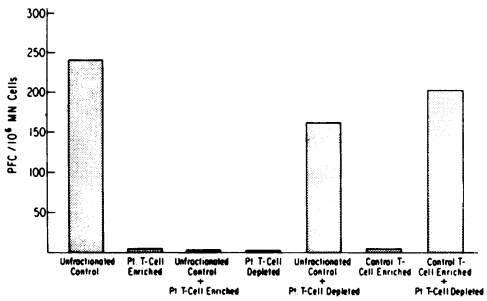

FIGURE 3 Numbers of plaque-forming cells (PFC) generated in co-cultures of various lymphocyte subpopulations from a normal control and those from a patient with common variable agammaglobulinemia. Pt., patient.

report, a good correlation was noted in co-culture studies when the plaque-forming cell assay was compared with the immunoglobulin biosynthesis assay for its ability to detect excessive suppressor cell activity in immunodeficient patients. This indicates the additional potential usefulness of the plaque-forming cell assay in the study of cell-cell interactions in immunodeficient and other patient populations.

The results of our studies of in vitro IgM biosynthesis and IgM anti-SRBC production in the immunodeficient patient population studied are in general agreement with the findings of others who have studied immunoglobulin synthesis in immunodeficient patients. Most patients with common variable and all patients with x-linked agammaglobulinemia have been found incapable of synthesizing significant quantities of immunoglobulin in vitro when their blood cells were stimulated with the polyclonal B-cell activator, pokeweed mitogen (3–7, 11, 13, 31). Only 3 of our 15 patients with common variable agammaglobulinemia, none of the 7 with x-linked agammaglobulinemia, and neither of the 2 with x-linked immunodeficiency with hyper-IgM made detectable plaque-forming cells.

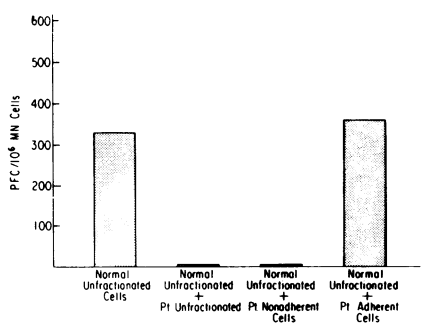

FIGURE 4 Numbers of plaque-forming cells (PFC) generated in co-cultures of unfractionated mononuclear blood cells from a normal individual and monocyte-depleted and -enriched mononuclear cell subpopulations from a patient with common variable agammaglobulinemia. Pt., patient.

TABLE IV
DNA Synthetic Responses to Pokeweed Mitogen by Mononuclear Cells from Patients with Primary Immunodeficiency Disorders

Disorder	N	Range*	Mean
		% of control	
CVAγ	15	20–327	89
XAγ	6	21–144	62
HypM	2	86–155	120

Abbreviations used in this table: CVAγ, common variable agammaglobulinemia; HypM, immunodeficiency with hyper-IgM; XAγ, x-linked agammaglobulinemia.
* Normalized values were computed using the following formula: response = (cpm [stimulated] − cpm [unstimulated] patient × 100)/(cpm [stimulated] − cpm [unstimulated] normal control).

None of the patients synthesized significant quantities of IgM in the biosynthesis assay. In addition, marked heterogeneity was noted among the three categories of immunodeficient patients that we evaluated in the capacity of individual patient's cells to suppress immunoglobulin synthesis and plaque-forming cell induction by normal mononuclear blood cells. Again, this is in keeping with reports by other investigators, because only 9 of 13 patients with common variable agammaglobulinemia studied by Waldmann et al. (4), all of 8 evaluated by Siegel et al. (5), and none of 14 investigated by De La Concho et al. (31) were found capable of causing significant suppression of immunoglobulin synthesis by normal cells. That three of our seven patients with x-linked agammaglobulinemia demonstrated excessive suppressor cell activity in co-cultures with normal cells in both assays was somewhat surprising, but a similar finding was noted in two of four patients with this defect studied by Waldmann and co-workers (11). Most unexpected, however, was the finding that cells from both patients with x-linked immunodeficiency with hyper-IgM, both of whom had markedly elevated serum concentrations of IgM, suppressed significantly both IgM synthesis and generation of plaque-forming cells by normal mononuclear cells.

In the only other reported study of the capacity of immunodeficient patients' blood cells to form hemolytic plaques in vitro, Dosch et al. (12) found that blood cells from four of five patients with x-linked agammaglobulinemia generated significant numbers of plaque-forming cells in response to in vitro stimulation with SRBC or ovalbumin. The addition of pokeweed mitogen to the antigen stimulated cultures inhibited the formation of plaques by patient cells but enhanced the development of plaque-forming cells by normal cells. Those authors hypothesized that surface

immunoglobulin negative B-cell precursors are present in the circulation of a majority of patients with this defect and, in support of this hypothesis, referred to the work of others demonstrating that patients with x-linked agammaglobulinemia have normal numbers of pre-B cells in their bone marrow (32). Dosch et al. (12) also postulated that the presence of excessive suppressor cell activity in their x-linked agammaglobulinemics was made more apparent by the addition of pokeweed mitogen. However, they failed to perform co-cultures between normal and patient cells to determine whether excessive suppressor cell activity could be demonstrated (12). As noted above, we failed to find any plaque-forming cells when mononuclear cells from the seven patients in the present study with x-linked agammaglobulinemia were cultured individually. The assay used in our studies differed from that of Dosch et al. (12) in that our cells were not cultured in the presence of antigen. We, like Fauci and Pratt (15), have been unable to obtain reproducible plaques when normal or patient mononuclear cells were cultured with SRBC in the absence of pokeweed mitogen.

In addition to evaluating the plaque-forming cell assay for its usefulness in quantifying the in vitro IgM antibody-forming capacity of patients with humoral immunodeficiency and in detecting excessive suppressor cell activity in their mononuclear blood cells, we explored its value as a tool to investigate regulatory phenomena involved in B-cell activation. Similar to the findings of others with immunoglobulin biosynthesis methods (3), our plaque-forming studies of cultured mononuclear cell subpopulations demonstrated that the cells from common variable agammaglobulinemics responsible for suppression of immunoglobulin synthesis by normal B cells are T cells. Adherent cells from two patients with common variable agammaglobulinemia whose T cells suppressed normal cell plaque formation failed to suppress under similar circumstances. Cells from two of the eight common variable agammaglobulinemic patients in our study who failed to demonstrate excessive suppressor cell activity by either assay were separated into T-enriched and T-depleted populations. The T-enriched fractions still failed to suppress normal cell plaque formation and the T-depleted populations failed to produce IgM anti-SRBC antibody when co-cultured with normal T cells. The latter findings suggest, as hypothesized by others (31), that an intrinsic B cell defect exists in such patients.

The role or roles of excessive T suppressor cell activity in patients with humoral immunodeficiency remains unclear. Perhaps the most convincing evidence that suppressor T cells may have a role in the genesis of the immunodeficiency comes from cell separation studies done by Waldmann et al. (11) and from studies in the present report showing that, for certain common variable agammaglobulinemics, removal of the patient's own T cells allowed his B cells to synthesize immunoglobulin or to form plaques. Neither of these studies excluded the possibility of a primary T helper cell defect, however. Against a primary role for excessive suppressor cell activity in these defects are the observations from the present study that (*a*) cells from only one of identical twins with common variable agammaglobulinemia caused significant suppression of IgM biosynthesis or plaque formation by normal cells, (*b*) that three of seven patients with x-linked agammaglobulinemia who lacked surface immunoglobulin positive B cells had excessive suppressive cell activity, and (*c*) that cells from both patients with x-linked immunodeficiency with hyper-IgM caused significant suppression of normal B-cell activation in both assays. In all of these latter situations, it would seem most likely that the increased suppressor cell activity develops secondarily to whatever the primary biologic defects are in such patients.

The factor or factors leading to development of increased suppressor cell activity are unknown. Recently it has been suggested that aggregated gamma globulin present in intramuscular immune serum globulin preparations could interact with the Fc receptor for IgG present on suppressor T cells, stimulating them for increased activity (33). This does not appear to be a likely explanation for the heterogeneity seen in the present study, because as many patients received plasma or the experimental intravenous immune serum globulin preparation as received intramuscular gamma globulin among those exhibiting increased suppressor cell activity. Infection is another possible factor leading to generation of increased suppressor cell activity. In this regard, it is of note that all three patients with x-linked agammaglobulinemia exhibiting this activity had persistent echovirus meningoencephalitis, whereas those not exhibiting it did not. Whether this increased suppressor activity contributed in any way to the patients' inability to get rid of the virus is unknown.

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