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## An ELISA spot assay for quantitation of human immunoglobulin-secreting cells

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*The elucidation of changes in populations of immunoglobulin-secreting cells has been a cumbersome process. We present a simplified method for the enumeration of human immunoglobulin-secreting cells with an ELISA spot assay (ESA). This method is specific, will detect all isotypes, including IgE, and is sensitive, detecting as little as 10 pg of antibody secreted. In this article, we describe the methodology for performing the ESA, demonstrate the kinetics for optimal use in both B-lymphoblastoid lines and fresh B cells, and determine the correlation between the ESA and immunoglobulin secretion under various experimental conditions. This assay permits an estimate of the level of immunoglobulin secreted per cell, thus distinguishing expansion of immunoglobulin-secreting cell populations from increases in average (per cell) immunoglobulin production. The combination of ESA and quantitation of immunoglobulin in supernatants of cultured cells provides an easy and reliable means for studying the regulation of immunoglobulin secretion. (J ALLERGY CLIN IMMUNOL 1991;88:235-43.)*

**Key words:** Antibody-secreting cells, isotype detection, ELISA spot assay

To understand the role of cytokines, mediators, or mitogens in the regulation of immunoglobulin secretion, it is becoming increasingly important to quantitate the number of B-lymphocytes that secrete specific isotypes of immunoglobulin in addition to studying the net effect of different culture conditions on immunoglobulin levels in culture supernatants. ELISA

### Abbreviations used

|      |                                               |
|------|-----------------------------------------------|
| EBV: | Epstein-Barr virus                            |
| ESA: | ELISA spot assay                              |
| SAC: | <i>Staphylococcus aureus</i> , Cowan strain I |
| MNC: | Mononuclear cells                             |
| PAF: | Platelet-activating factor                    |
| BSA: | Bovine serum albumin                          |
| Ab:  | Antibody                                      |

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detection of immunoglobulin secreted into cell-culture supernatants provides data without distinguishing whether mitogenic stimulation increases the number of cells secreting immunoglobulin or enhances the amount of immunoglobulin secreted per cell. The enumeration of single cells secreting specific immunoglobulin isotypes has, to date, been a cumbersome process. Single-cell assays, such as the plaque-forming assay<sup>1</sup> or limiting-dilution techniques,<sup>2</sup> may aid in discerning single-cell immunoglobulin secretion but are somewhat limited. The limiting-dilution technique is an elegant but time-consuming method that

suffers from the needs for a feeder cell and a B cell mitogen (e.g., EBV) to stimulate immunoglobulin secretion.<sup>2</sup> The reverse plaque-forming assay is an indirect measure of immunoglobulin-secreting cells but has been difficult to adapt to isotypes other than IgM.<sup>1</sup>

To meet the need for a rapid, sensitive, and specific method to quantitate immunoglobulin-producing cells in humans, we have developed a technique with the principles of ELISA. This assay permits the enumeration of immunoglobulin-secreting cells of different isotypes rapidly and reliably and allows for easy manipulation of experimental conditions. This approach was first described for murine cells by Sedgwick and Holt,<sup>3</sup> and later adapted for human IgG and IgE isotypes.<sup>4</sup> To date, it has not been fully exploited for assessment of the rapidly growing number of mediators and cytokines that may stimulate human immunoglobulin-producing cells.

We present here an adaptation of the ESA for human immunoglobulin-secreting cells and document isotype specificity and the sensitivity of this procedure. These data we present define the sensitivity, specificity, and reproducibility of the ESA. In addition, we describe the capability to detect all four human immunoglobulin isotypes, including IgE. We demonstrate the correlation with total immunoglobulin measured in bulk culture supernatants and suggest applications of this technique in investigation of the regulation of immunoglobulin synthesis.

## MATERIAL AND METHODS

### Cell lines

The human B-lymphoblastoid cell line, HSCE<sup>-</sup>, is an IgG-secreting line resulting from the spontaneous transformation of EBV-infected tonsillar B-lymphocytes. The U266 cell line is an IgE-secreting B cell line that is of myeloma lineage. The Kirkland III cell line (kindly provided by Dr. J. F. Jones, Denver, Colo.) is an IgM-secreting B cell line established by the spontaneous transformation of peripheral blood cells from a patient with a chronic-active EBV infection. The LA350 cell line (kindly provided by Drs. H. Rosenblatt and W. Shearer, Houston, Texas) is an IgM-secreting EBV-transformed B cell line. All four cell lines were maintained in culture in complete medium, consisting of RPMI 1640 (Gibco, Grand Island, N.Y.) and 10% fetal calf serum (Hyclone Laboratories, Logan, Utah) supplemented with 4 mmol/L of L-glutamine, 100 U/ml of penicillin, 100 µg/ml of streptomycin, and 1 mmol/L of sodium pyruvate (Gibco).

### Tonsillar MNCs

Human tonsils were obtained after surgical removal. Tonsils were placed in complete medium supplemented with 1 µg/ml of amphotericin B (Fungizone; Gibco, Grand Island, N.Y.) and were processed within 1 hour of surgery. Tissue was manually minced and washed three times in RPMI 1640 supplemented with 2% fetal calf serum. MNCs

were prepared by Ficoll-Hypaque gradient centrifugation (Pharmacia, Uppsala, Sweden).<sup>5</sup>

### Cell culture

Cell lines were cultured in a complete medium at a concentration of  $1 \times 10^5$  cells per well in a humidified incubator at 37° C, 5% CO<sub>2</sub>, in 96-well, round-bottom plates (Corning Glass Works, Corning, N.Y.). MNCs were resuspended in fresh complete medium at  $1 \times 10^6$  cells per milliliter and cultured in 24-well plates (Falcon, Lincoln Park, N.J.). In selected experiments, SAC (Calbiochem, San Diego, Calif.), prepared as previously described,<sup>6</sup> or PAF (1-O-hexadecyl-2-O-acetyl-*sn*-glycero-3-phosphorylcholine) (Biomol Research Laboratories, Plymouth Meeting, Pa.) was added. Supernatants from cell cultures were harvested and frozen at -20° C until they were used in the ELISA assay. Cells were recovered from the culture plates, washed three times in wash medium, and then applied to specific isotype-coated plates for the ESA.

### Measurement of immunoglobulin synthesis by ELISA

Round-bottom, 96-well ELISA plates (Nunc, Roskilde, Denmark) were coated with 3 µg/ml of goat antihuman Ab to either IgG, IgM, IgA, or IgE (Tago, Inc., Burlingame, Calif.) and blocked with 0.1 mol/L of Tris buffer supplemented with 1% BSA. Supernatant was appropriately diluted in 0.1 mol/L of Tris buffer supplemented with 1% BSA. After an overnight incubation at 4° C, the plates were washed three times, and goat antihuman Ab conjugated with alkaline phosphatase (Tago, Inc.) was added, diluted to the optimal concentration with 0.1 mol/L of Tris and 1% BSA. After a 2-hour incubation, plates were developed with a 2 mmol/L solution of paranitrophenol phosphate (Sigma Chemical Co., St. Louis, Mo.) in 1 mol/L of Tris buffer. Colorimetric analysis at wavelength 410 nm was performed with an automated ELISA reader (Bio-Rad, Inc., Pleasanton, Calif.). Cell-culture immunoglobulin values were compared to a standard curve generated from serial dilutions of standard sera (Atlantic Antibodies, Scarborough, Me.). Analysis of ELISA data was performed with the Microplate Manager software for the Macintosh computer (Bio-Rad).

### ESA

The ESA for enumeration of human immunoglobulin-secreting cells was performed by modification of the method according to that of Sedgwick and Holt.<sup>3</sup> Briefly, 96-well, flat-bottom ELISA plates (Corning Glass Works) were coated with 3 µg/ml of goat antihuman IgG, IgM, IgA, or IgE (Tago, Inc.). Cells were added to the plates and incubated in a humidified incubator with 5% CO<sub>2</sub> at 37° C. Cells were then removed from the plates by washing three times with water, and goat antihuman immunoglobulin, linked to alkaline phosphatase (Tago, Inc.), was added at the optimal concentration. After a 2-hour incubation at 37° C, the plates were washed, and the phosphate substrate was added as a combination of 5-bromo-4-chloro-3-indoyl phosphate and 1.2% agarose (both purchased from Sigma Chemical Co.). The plates were incubated at 37° C for 1 hour and then at

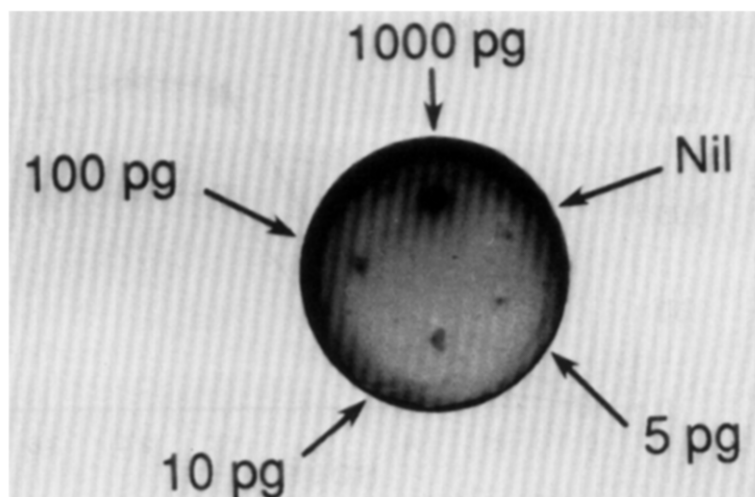


**FIG. 1.** Sensitivity of ESA. A microtiter well on an ESA plate was coated with 50  $\mu$ l of agarose, and serial dilutions of a known standard IgE were injected into the agarose. The plate was then developed as described in MATERIAL AND METHODS. The limit of reliable detection for IgE was 10 pg/ml. Similar resolution was achieved with IgG and IgM (data not presented). Results presented here are one of three representative experiments.

room temperature overnight. Spots were hand counted under microscopy.

### Calculation of immunoglobulin secreted per spot

Before plating on ESA plates, washed and cultured B cells were counted by Coulter counter (Coulter Electronics, Hialeah, Fla.). Equal numbers of cells were plated in each well of the ESA plate. As cells for measurement of immunoglobulin production were plated at  $1 \times 10^5$  to  $1 \times 10^6$  cells per well and ESA was performed with  $10^3$  to  $10^5$  cells per well, per cell immunoglobulin production was calculated with the following formula:

$$k \left( \frac{\text{Ig}}{\text{No. spots}} \cdot \frac{1}{n} \right)$$

Where  $k$  = final number of cells in culture/number of cells originally cultured and  $n$  = cell number originally cultured for detection of immunoglobulin (Ig) in supernatants/cell number plated for ESA.

### Statistics

Statistical analysis was performed with the Statview 512+ program (BrainPower, Inc., Calabasas, Calif.) for the Macintosh computer. Regressions were performed for assessment of correlation of ESA and supernatant results, and  $p$  values were obtained with Student's  $t$  test.

## RESULTS

### Isotype specificity of the ESA

To establish the specificity of detection of immunoglobulin isotypes by the ESA technique, B-lymphoblastoid cell lines that secrete specific immu-

noglobulins were plated on the appropriately coated spot-assay plates, as well as on plates that were coated for other isotypes. In these studies, we used the IgG-secreting B cell line, HSCE<sup>-</sup>, the IgM-secreting B cell line, Kirkland III, and the IgE-secreting B cell line, U266; as a negative control to assess nonspecific spot formation, the nonsecreting Raji B cell line was also used. Included on the ELISA plates were serial dilutions of standardized sera that created a diffuse blue coloring in the wells when they were developed with the 5-bromo-4-chloro-3-indoyl phosphate and agarose solution. The number of cells secreting detectable amounts of immunoglobulin at any point in time in these B-lymphoblastoid lines was high (3% to 15%); therefore, low numbers of cells generally were plated. The optimal number was  $10^3$  cells per well for all cell lines. Washed cells were plated and incubated for 16 to 18 hours to assess spontaneous immunoglobulin production. The assay was specific in the detection of the specific isotype alone for the IgG-, IgM-, and IgE-secreting cells. This finding was confirmed by use of standard monoclonal sera on the ESA plates that blocked the appearance of spots only of the isotype to which the Ab was directed. In addition, immunoglobulin secreted from each cell line bound only to plates coated for their own corresponding isotype, and no spots were detected when specific immunoglobulin-secreting cells were incubated on plates coated with a different isotype, further confirming the specificity of the assay. Finally, the Raji cell line did not cause any nonspecific spot formation, even when it was plated at  $10^5$  cells per well.

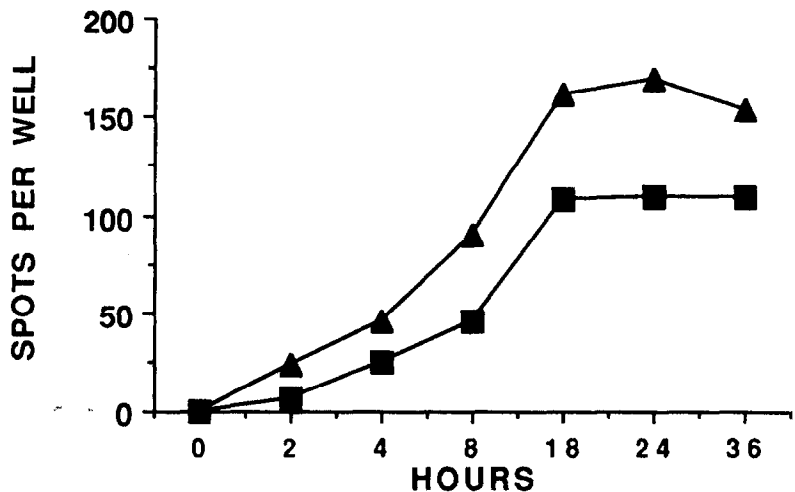


**FIG. 2.** Kinetics of ESA. HSCE<sup>-</sup> cells (▲) or fresh tonsillar MNCs (■) were plated for various times on an IgG-coated ESA plate and developed as described in MATERIAL AND METHODS. The number of spots detected plateaus at approximately 16 to 18 hours of incubation, after which clarity of the spots decreased. Identical results were obtained with U266 and Kirkland III cell lines. Results of one of four representative experiments are presented.

**TABLE I.** Serial dilution of immunoglobulin-secreting B-lymphoblastoid lines

| Cell line               | Spots/well      |                 |                     |                     |                 |    |
|-------------------------|-----------------|-----------------|---------------------|---------------------|-----------------|----|
|                         | 10 <sup>4</sup> | 10 <sup>3</sup> | 5 × 10 <sup>2</sup> | 2 × 10 <sup>2</sup> | 10 <sup>2</sup> | 10 |
| LA350                   | *               | 55 ± 4          | 26 ± 6              | 19 ± 7              | 7.6 ± 1         | 0  |
| HSCE <sup>-</sup> (IgG) | *               | 55 ± 15         | 29 ± 14             | 15 ± 6              | 8 ± 3           | 0  |
| U266 (IgE)              | *               | 33 ± 6          | 19 ± 7              | 7 ± 4               | 3 ± 1           | 0  |

Immunoglobulin-secreting cell lines were washed three times in RPMI and plated at the indicated concentrations on isotype-specific ESA plates coated with anti-IgM, anti-IgG, or anti-IgE. After development, the spots in each well were counted.

Results of four experiments are presented (mean ± SEM).

\*Confluence of spots prevented accurate spot enumeration.

### Sensitivity of the ESA

The limit of immunoglobulin detection was examined by injection of 10 µl of decreasing dilutions of a known standard IgE (Tago, Inc.) onto an ESA well, precoated with agarose (Fig. 1). This was done to simulate the microenvironment of a cell and to determine the minimum amount of immunoglobulin secreted that can be detected as a distinct spot. As indicated in Fig. 1, the limit of detection of IgE was as low as 10 pg. Lower concentrations of the IgE or diluent buffer failed to develop distinct spot formation. Spot formation by higher concentrations of IgE were both larger and more distinct. Similar levels of sensitivity were detected for IgG and IgM (data not presented).

An additional advantage of the ESA is the low number of cells that is required for accurate detection of spots. As will be discussed, accurate spot formation was achieved by immunoglobulin-secreting cell lines with as few as 10<sup>2</sup> cells per well (Table I).

### Kinetics of the ESA

To best detect the effects of mitogens or cytokines on cells, the temporal relationship of the assay to the addition of stimulus is crucial. Once the optimal time for the cells to respond to a stimulus has been determined, the spot assay should provide fairly rapid information as to the effect of the stimulus on the stimulated cell population. We determined the optimal time of assay development for detection of the maximum number of distinct spots. As indicated in Fig. 2, the detection of immunoglobulin-secreting cells was noted as early as 2 hours after addition of the cells to coated plates. The maximum number of spots was detected 16 to 18 hours after cell plating. Beyond this time, the number of spots did not increase, but they became larger and less distinct. This may be due to cell division (24 to 36 hours) or binding of increasing amounts of immunoglobulin to the plates. The kinetics of spot appearance were virtually identical for the HSCE<sup>-</sup> IgG-secreting cell line (Fig. 2), as well

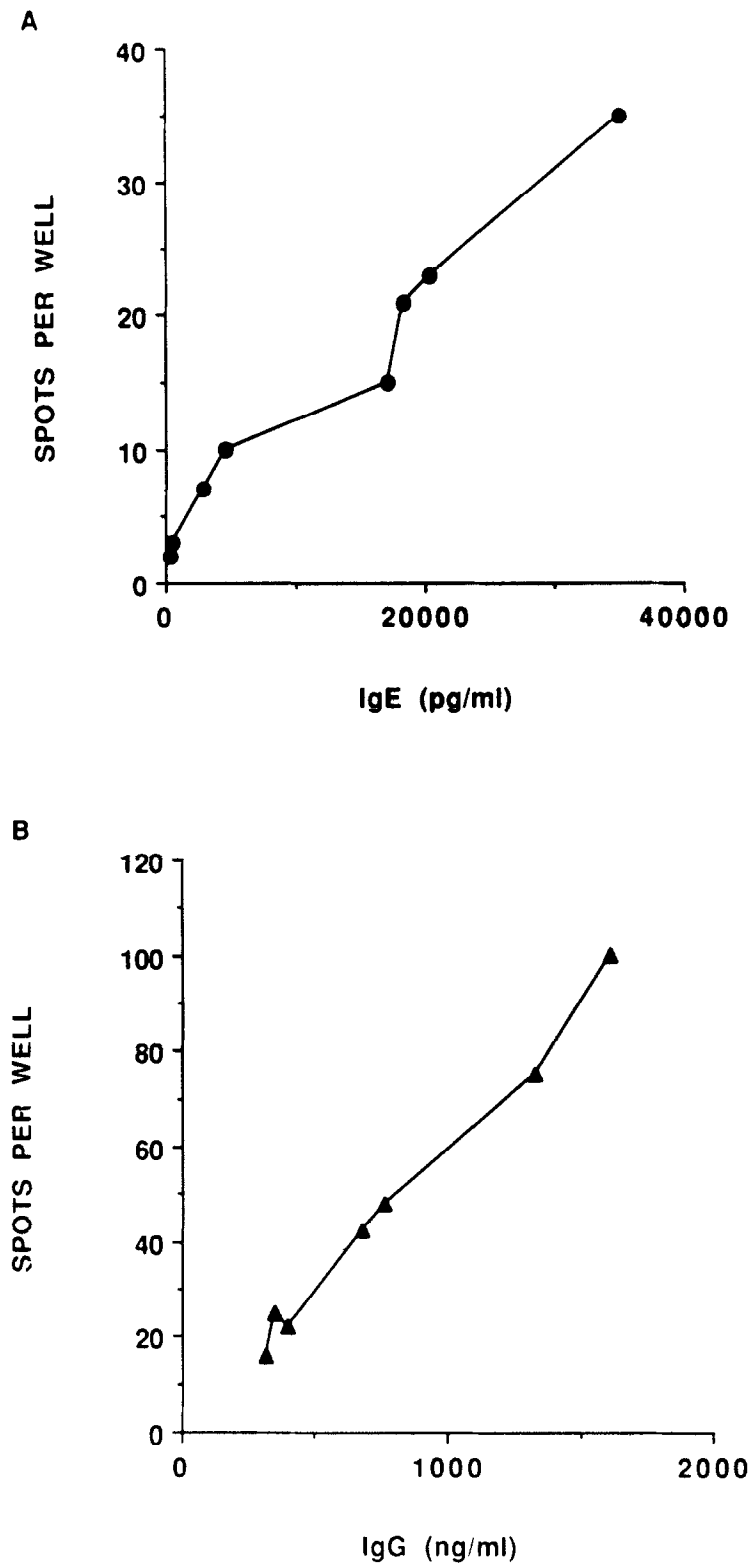


**FIG. 3.** Correlation between ESA and immunoglobulin secretion. Tonsillar MNCs from seven to eight individual donors were plated at  $1 \times 10^6/\text{ml}$  for 7 days to assess spontaneous IgG and IgE production. After 7 days, supernatants were harvested, and the cells were washed three times, plated on IgG- or IgE-coated ESA plates, and incubated for 16 hours. **A**, Comparison of IgE ( $N = 8$ ) detected in supernatants versus number of spots ( $r = 0.975$ ). **B**, IgG comparison ( $N = 7$ ) ( $r = 0.987$ ).

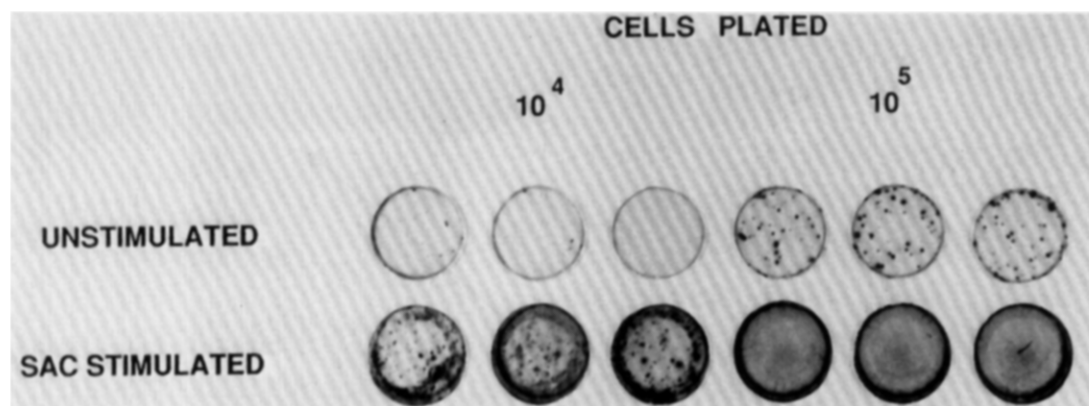


**FIG. 4.** SAC stimulation of IgM-secreting cells. MNCs ( $10^6/\text{ml}$ ) were incubated in the presence or absence of SAC. Washed cells were plated at  $10^4$  or  $10^5$  cells per well, and spots were developed overnight, as described in MATERIAL AND METHODS.

as the U266 and Kirkland III cell lines (data not presented).

#### **Correlation of spot detection to numbers of cells plated**

We next determined the correlation between plating serial dilutions of the immunoglobulin-secreting cell lines and the numbers of spots detected. ESA plates were prepared for individual isotypes, and the cell lines were plated after a 24-hour incubation in fresh complete medium at serial log concentrations ranging from  $10^1$  to  $10^4$  cells per well. Spots were detected most easily when they were plated at  $10^2$  or  $10^3$  cells per well. Plating  $>10^3$  cells from the immunoglobulin-secreting lymphoblastoid lines led to a diffuse, indistinct, blue coloration of the wells; lower numbers of cells ( $<100$ ) failed to consistently result in detectable spots. As described in Table I, the detection of spots from all isotypes was clearly correlated with numbers of cells plated.

#### **Correlation with immunoglobulin production by stimulated tonsillar B-lymphocytes**

In addition to the study of immunoglobulin-secreting B-lymphoblastoid cell lines, the optimal conditions for detection of single-cell Ab production by fresh B-lymphocytes were established. For these studies, tonsillar mononuclear lymphocytes were used and were compared to immunoglobulin-secreting B-lymphoblastoid lines. As illustrated in Fig. 2, the kinetics of spot development were found to be identical in these two cell populations. As was expected, a higher number of tonsillar cells were needed to be plated to detect spot formation reproducibly. Depending on the isotype,  $10^4$  to  $10^5$  cells were found to be

optimal for detecting a sufficient quantity of spots reproducibly.

The ESA demonstrated a good linear correlation between numbers of spots detected and the amount of immunoglobulin detected in supernatants from cultured tonsillar cells. The comparison of spontaneous immunoglobulin production from tonsillar lymphocytes, obtained from seven to eight different subjects, is indicated in Fig. 3. ESA was performed in parallel to supernatant immunoglobulin levels for IgG and IgE in each donor after a 7-day culture. Supernatant IgE levels, plotted against the number of IgE-producing cells detected after a 16-hour incubation on an ESA plate is illustrated in Fig. 3, A. The detection of IgE in the cell-culture supernatants strongly correlated with the number of spots detected ( $r = 0.975$ ). Data for IgG production (Fig. 3, B) illustrate a similar strong correlation between spots detected and levels of IgG measured in culture supernatants ( $r = 0.98$ ).

#### **Applications of the ESA**

To be maximally useful for studying human immunoglobulin production, the ESA must be able to detect all four isotypes reliably. An additional, important application is the ability to differentiate between increases in immunoglobulin production that may be due to an increase in immunoglobulin-secreting cell number and/or increase due to augmentation of immunoglobulin production by individual cells. To approach the latter question, we used the polyclonal B cell stimulus, SAC, and PAF to delineate the ability of the ESA to distinguish these alternatives. MNCs from tonsils were cultured for 8 days, with and without a 1:10000 vol/vol dilution of SAC, and analyzed for the production of all four isotypes, IgG, IgA, IgM, and IgE. SAC is known to stimulate B cell prolifer-

TABLE II. SAC stimulation of tonsillar MNCs

| Condition    | Isotype | Ig (pg/well)             | Spots/well   | Ratio (pg/spots) |
|--------------|---------|--------------------------|--------------|------------------|
| Unstimulated | IgG     | $75 \times 10^3 \pm 16$  | $36 \pm 18$  | $206.0 \pm 80$   |
|              | IgA     | $60 \times 10^3 \pm 12$  | $24 \pm 2$   | $255.0 \pm 60$   |
|              | IgM     | $370 \times 10^3 \pm 16$ | $108 \pm 14$ | $342.6 \pm 50$   |
|              | IgE     | 0                        | 0            | 0                |
| SAC          | IgG     | $169 \times 10^3 \pm 25$ | $104 \pm 20$ | $24.4 \pm 6$     |
|              | IgA     | $432 \times 10^3 \pm 47$ | $40 \pm 12$  | $162 \pm 30$     |
|              | IgM     | $954 \times 10^3 \pm 14$ | $291 \pm 18$ | $49.2 \pm 15$    |
|              | IgE     | $31 \times 10^3 \pm 8$   | $10 \pm 3$   | $46.5 \pm 12$    |

Ig, Immunoglobulin.  
MNCs ( $10^6$ /ml) were incubated for 7 days in the presence or absence of SAC. Supernatants were collected, and immunoglobulin was quantitated. Washed cells were plated at  $10^4$  to  $10^5$  cells/well in the ESA. Ratio (picograms per spot) was calculated as per the formula described in MATERIAL AND METHODS with  $k = 1$  and  $n = 10$  for unstimulated cells;  $k = 1.5$  and  $n = 100$  for stimulated cells. Results of three experiments are presented (mean  $\pm$  SEM).

TABLE III. Effect of PAF on B-lymphoblastoid lines

| Cell line         | Unstimulated             |             |                 | PAF stimulated            |             |                 |
|-------------------|--------------------------|-------------|-----------------|---------------------------|-------------|-----------------|
|                   | Ig (pg/well)             | Spots/well  | Ratio (pg/spot) | Ig (pg/well)              | Spots/well  | Ratio (pg/spot) |
| HSCE <sup>-</sup> | $3 \times 10^3 \pm 50$   | $45 \pm 5$  | 0.66            | $47 \times 10^3 \pm 400$  | $68 \pm 8$  | 6.9             |
| U266              | $30 \times 10^3 \pm 150$ | $235 \pm 2$ | 1.27            | $330 \times 10^3 \pm 350$ | $292 \pm 3$ | 11.3            |

B-lymphoblastoid lines were cultured at  $1 \times 10^5$  cells per well, with or without  $10^{-9}$  mol/L of PAF for 24 hours, washed three times, and then plated at  $10^3$  cells per well on an ESA plate and incubated for 16 hours. Supernatants for immunoglobulin production from cultures were run simultaneously with the ESA. There was no difference in cell number at 24 hours in PAF-treated or untreated cells. Ratio (picograms per spot) was calculated as per the formula described in MATERIAL AND METHODS with  $k = 1.2$  and  $n = 100$ . Results of three experiments are presented (mean  $\pm$  SEM).

ation and increase immunoglobulin production of all isotypes.<sup>6</sup> An example of these changes detected in the ESA are illustrated in Fig. 4. As illustrated in Fig. 4, to detect spontaneous IgM production,  $10^5$  cells per well were plated with mean spot number of  $108 \pm 14$  spots per well detected. After stimulation with SAC, plating of a similar number of cells resulted in a confluence of spots. Plating tenfold fewer cells ( $10^4$  per well) resulted in a mean of  $291 \pm 18$  spots per well detected.

A summary of the results of three experiments are presented in Table II. SAC stimulation resulted in a marked increase in immunoglobulin production of all isotypes, as measured in culture supernatants. This finding was paralleled by a dramatic increase in numbers of spots detected for all isotypes as well. Because, at the end of the 7-day culture period, there was a 50% increase in cell numbers in stimulated versus unstimulated cultures, the resulting calculation of picograms per spot reflected a mean decrease in immunoglobulin secretion per cell.

Different conclusions were drawn when lymphoblastoid cells were incubated with PAF. We previously

reported that PAF enhances the production of immunoglobulin from B-lymphoblastoid cell lines.<sup>7</sup> The detection of immunoglobulin-producing cells by ESA compared to immunoglobulin levels in cell-culture supernatants after a 24-hour incubation in the presence or absence of  $10^{-9}$  mol/L of PAF is presented in Table III. The large increase in immunoglobulin detected in the supernatants does not parallel the change in numbers of immunoglobulin-secreting cells. When the picogram per spot ratio is calculated, these data strongly suggest the action of PAF is via stimulation of individual cell secretion of immunoglobulin.

DISCUSSION

ESA was described in 1983 by Sedgwick and Holt<sup>3</sup> and Czerkinsky et al.<sup>8</sup> as a solid-phase technique for detecting specific Abs in immunized mice. The procedure has been adapted for determination of specific Abs in mice<sup>9-11</sup> and humans<sup>12-15</sup> and for the enumeration of cytokine-secreting cells.<sup>16-18</sup>

Regulation of immunoglobulin synthesis has become increasingly complex as the recognition of cytokines and mediators increases. The understanding

of the effects of secreted cell products and drugs on the ability of cultured lymphocytes to secrete immunoglobulin can now only be accomplished if frequencies of immunoglobulin-secreting cells can be performed in parallel to quantitation of immunoglobulin secretion. To approach these issues, we have adapted the ESA to study the regulation of immunoglobulin secretion in immunoglobulin-secreting lines as well as in fresh B cell populations. The specificity, sensitivity, and kinetics of this assay for cell lines and fresh B cells are presented in this article and confirm the ability to distinguish increases in total immunoglobulin secretion as a function of the numbers of immunoglobulin-secreting cells as well as differences in the amount of immunoglobulin secreted by an individual cell.

Secretion of all isotypes of immunoglobulin can be detected in this ESA. Abs secreted by single isotype-producing lymphoblastoid cell lines were specifically detected, and IgG, IgM, and IgE were only detected on plates specifically coated for these isotypes. No nonspecific binding of other secreted proteins or other isotypes was detected on the specifically coated plates. Because the cells are secreting directly onto the Ab-coated plate, the likelihood of detecting small quantities of Ab is enhanced. Although it is technically difficult to determine the exact amount of immunoglobulin that can be detected from a single cell or in a particular spot, these data indicate that an amount as low as 10 pg can be clearly visualized as a spot. This heightened sensitivity makes it possible to determine subtle differences in immunoglobulin secretion by single cells and bulk cell populations.

The assay is rapid, with optimal recovery and resolution of spots being approximately 16 to 18 hours after plating the cells. The speed of detection is particularly useful in the direct assessment of the action of a mediator (e.g., PAF) or mitogen (e.g., SAC) on activated cells. The ESA will detect immunoglobulin secretion by a population before or within the span of a single-cell division.

A major advantage of this technique is the ability to study the response of immunoglobulin-secreting cells to a particular stimulus without the need for addition of other mitogenic substances to the cells. Previous studies evaluating the frequency of immunoglobulin-secreting cells have been hampered by the absence of a simple and direct technique of assessment. In the limiting-dilution technique,<sup>2</sup> for example, cells are carried in culture by the addition of EBV and a feeder cell line; supernatants are used to detect wells positive or negative for an immunoglobulin isotype. The results are highly variable, require many replicates, and are entirely dependent on the frequency of B cells transformed by EBV. The ESA permits the

direct examination of cell populations. No new or different mitogens are added to the wells, and as noted, the optimal time for development of the spots is well within the limits of one-cell division. This is critical for the accurate assessment of the effect of a substance added to cultured cells. The ESA is therefore able to differentiate between a substance that enhances cell division, causing more cells to secrete immunoglobulin, and a stimulus that enhances the per cell secretion of immunoglobulin with or without clonal expansion.

The effects of SAC and PAF on immunoglobulin-secreting cells provide contrasting examples for alternatives that result in enhancement of immunoglobulin production. Addition of SAC resulted in expansion of the number of tonsillar B cells that were capable of secreting immunoglobulin. In contrast, PAF augmented the production of IgE and IgG by transformed B-lymphocyte lines by enhancing the per cell production of immunoglobulin without significantly altering numbers of immunoglobulin-secreting cells.

IgE is generally more difficult to detect because of the low frequency of IgE-secreting cells in preparations of tonsillar or peripheral blood B cells.<sup>19</sup> The expansion of the IgE-secreting cell population by the polyclonal stimulus SAC was clearly detected in the ESA (Table II). Similar results have also been demonstrated after addition of interleukin-4 to preparation of tonsillar MNCs.<sup>20</sup>

There is as yet no good method for linking the size of the spots to the amount of immunoglobulin secreted. With 24-well plates and a brief 2- to 4-hour cell incubation, Holt et al.<sup>21</sup> contrasted spot size in primary immunized rats to that of the secondary response to ovalbumin. They measured ESA plaque diameter and suggested that, since more large plaques were found in the secondary responders, the larger plaques may reflect changes in net Ab production per cell. In addition, a larger amount of immunoglobulin was detected in the supernatants from secondary responders. Unfortunately, data were not provided on the number of plaques per culture, making confirmation of the observations difficult. It is also likely that, since immunoglobulin secretion proceeds with longer incubation times, the size of the plaques do grow. This was indicated by the loss of spot clarity after 24 to 36 hours of incubation in our kinetic profiles (Fig. 2).

The estimate of a picogram-per-spot value for a given population is perhaps a less cumbersome approach than actual diameter measurements and likely more practical for 96-well plates. By comparing cell counts in control and stimulated populations, the spots detected, and the amount of immunoglobulin in the

supernatants, a value for immunoglobulin secretion per cell can be estimated.

As we extend our understanding of the complexity of the immune response, detection of single-cell responses will become increasingly important. This will be important in the evaluation of the effect of immunologic intervention both in vivo and in vitro. The ESA provides a flexible tool for elucidating single-cell kinetics of the response to an unlimited number of stimuli and provides a valuable addition to the evaluation of the regulatory influences on immunoglobulin secretion, including IgE production, in which the frequency of secreting cells is extremely low.

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