A RAPID, SEMI-AUTOMATED COUNTING PROCEDURE FOR ENUMERATION OF ANTIBODY-FORMING CELLS IN GEL AND NUCLEATED CELLS IN SUSPENSION[1]

By

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Running Title: Semi-Automated PFC Counting

FOOTNOTES

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INTRODUCTION

Since the description by Jerne *et al* (1) of the hemolysis-in-gel technique for enumerating single antibody-producing cells, this procedure has become one of standard usage in most laboratories engaged in immunological research. Different laboratories use a variety of modifications of the technique, whether in a gel support medium as initially described or the suspension technique described by Cunningham and Szenberg (2). Irrespective of the modification employed, the final analysis involves enumeration of plaques which have developed in the indicator erythrocyte suspension. Typically, this has been done visually by examining either slides or petri dishes with an appropriate light source either with or without the aid of a suitable magnifying lens or stereozoom microscope.

Since visual examination of oftentimes numerous specimens (slides or petri dishes) containing varying numbers of plaque-forming cells (PFC) can be both time consuming and fatiguing (thus contributing to potential counting error), we have modified a commercially available electronic bacterial colony counter (Fisher Count-All^{R}, Artek Systems, Inc., Farmingdale, New York) to allow rapid, semi-automated counting of PFC directly from the slides on which they are developed. In addition, the same machine has been adapted to allow rapid, semi-automated counting of nucleated cells in suspension directly from a conventional hemacytometer chamber. These modifications make it possible, therefore, to utilize a single machine to perform rapid, highly accurate enumeration in two very frequently employed procedures in most immunology research laboratories - *e.g.* routine nucleated cell counts and plaque-forming cell assays.

MATERIALS AND METHODS

Counting Apparatus.

The Fisher Count-All[R] instrument consists of a rapid scanning, high resolution television camera, electronic circuitry, dark field/bright field light source and digital readout all enclosed in relatively compact metal housing. An electronic processor unit capable of varying detection parameters sits beside the unit and a 23 or 30 cm. television monitor sits on top of the metal housing. The latter components are sufficient for purposes of enumerating plaque-forming cells on slides. If one wishes also to use the instrument for enumeration of nucleated cells from standard hemacytometer chamber, as reported here, then an auxiliary camera, supported by a camera mounting stand, is positioned on the vertical microscope tube of a triocular Zeiss photomicroscope; the auxiliary television camera then plugs into the counter.

The video signals from either of the television cameras are transmitted to the electronics unit and then processed to provide a single count for each object. Simultaneously, the video image is displayed on the television monitor. An electronic window is also displayed on the monitor, outlining the region within which objects are to be counted; the position and size of the window are adjustable such that its shape can be made either circular, square or rectangular. Within the window, objects whose optical density exceeds a manually set threshold are sensed and counted, and an illuminated dot or "flag" appears superimposed on each counted object on the video monitor display. Since the optimal threshold for accurate enumeration of optically dense objects will vary depending on a number of variables, including the amount of light reaching the television camera, etc., this ability to visually inspect the flagged objects on the video monitor permits the operator to reach a correct threshold setting quite easily each time the machine is used.

Antigens, Animals and Immunizations.

The antigens employed in these experiments were standard hapten-protein conjugates utilized routinely in our laboratory. Keyhole limpet hemocyanin (KLH) was purchased from Pacific Bio-Marine Supply Company, Venice, California. 2,4-dinitrophenyl (DNP) and benzylpenicilloyl (BPO) conjugates of KLH (DNP_8-KLH and BPO_{10}-KLH) were prepared as described in previous publications (3,4). An extract of *Ascaris suum* (ASC) was a gift of Dr. Kurt J. Bloch, Department of Medicine, Massachusetts General Hospital, Boston, Massachusetts; $DNP_{2.1}$-ASC was prepared as previously described (5).

Mice of the inbred lines BALB/c and (BALB/c x A/J)F_1 hybrids (CAF_1) were obtained from the Jackson Laboratory, Bar Harbor, Maine. Mice were immunized intraperitoneally (i.p.) with either 2 μg of DNP-KLH, 1 μg of BPO-KLH or 10 μg of DNP-ASC, adsorbed onto 4 mg. of aluminum hydroxide gel (alum); 3-4 weeks after primary immunization, mice were generally boosted i.p. with 5 μg of the original immunizing hapten-protein conjugate in saline. Such donor mice were then used as a source of spleen cells 1-3 weeks after the secondary boost and these donor cells were then either adoptively transferred into syngeneic, irradiated (700 R) recipients or utilized as a source of hapten-primed cells for *in vitro* culture utilizing the micro-culture technique described in a previous publication (6). In the adoptive transfer experiments, recipients of primed cells were challenged i.p. with the immunizing antigen utilizing the same dose employed for primary immunization, and the cells were obtained from the spleens from such recipients 7 days after secondary challenge for analysis in the plaque forming cell assay. In the case of *in vitro* culture, the cells were cultivated in micro-titer plates (0.5×10^6 cells per well) in the presence of the original immunizing antigen and then analyzed for PFC of the IgG class 4 days after initiation of the culture.

Determination of Anti-DNP and Anti-BPO Antibody-Producing PFC.

Single cell suspensions of either spleen cells from irradiated adoptive transfer mice or of cultured spleen cells from micro-titer culture wells, were prepared and appropriately washed in minimum essential medium. Anti-DNP and anti-BPO antibody-forming cells were assayed by a modification (7) of the PFC technique described by Jerne *et al* (1). Sheep erythrocytes (SRBC) conjugated with either 2,4,6-trinitrobenzenesulfonic acid (TNP,8) or with BPO (4) were used as indicators for DNP- and BPO-specific PFC, respectively. Indirect, or IgG, PFC were developed with a polyvalent rabbit anti-mouse immunoglobulin antiserum as previously described (7). At the completion of each assay, all slides were examined visually by one or more persons and then counted by the machine. The values reported here represent absolute counts of PFC per slide with no attempt to report conversions of such values into numbers of PFC per spleen or per 10^6 cells.

Enumerations of nucleated cell suspensions were carried out using appropriate dilutions of spleen cells suspended in 2% acetic acid and then dispensed onto standard hemacytometer chambers; chambers were then examined visually under the microscope and immediately thereafter displayed on the television monitor for machine counts.

RESULTS

Enumeration of Anti-DNP and Anti-BPO PFC.

Microscope slides overlaid with agarose containing medium, indicator TNP-SRBC or BPO-SRBC and suspensions of spleen cells or cultured cells, were placed in a specially designed plexiglass holder which was then placed into the visual field of the Count-All ^{R} machine. Using the dark field light source, an area of the slide

corresponding to 59% of the total slide area was outlined by the rectangular window. By outlining the middle area of the slide, errors resulting from optical deviations near the borders at the ends of each slide could be minimized. The area counted on each slide could be precisely set on different occasions since the electronic processor unit is capable of giving a digital readout of the area framed by windows of a given dimension. As stated in MATERIALS AND METHODS, each individual slide was examined visually by one or more persons in terms of enumerating 1) the numbers of PFC within the precise area counted by the machine (outlined by marking pen on the back of each slide), and 2) the total number of PFC present on each slide. These visual counts were then compared with the counts made by the machine. These data are compared in the form of a scatter diagram shown in Figure 1. Visual and instrument counts on 72 individual slides spanning a relatively wide range of total PFC per slide are presented here. The slope of the line drawn for these points is 1.01 and the correlation coefficient (r) is 0.995, thereby demonstrating excellent agreement between the visual and instrument counts.

The PFC indicated in Figure 1 were all of the IgG class and are not distinguished here by their specificity (*i.e.* anti-DNP or anti-BPO). In fact, no substantive difference was observed in the correlation between visual and instrument counts depending on the specificity of the PFC analyzed, thereby indicating that PFC of essentially any specificity could be expected to be accurately counted by this method. We did notice, however, that in order to detect all PFC on any given slide, it was necessary to vary the threshold setting depending on whether the PFC were either very large, very small, sharply demarcated or not so sharply demarcated. For example, the clarity of direct (IgM) and indirect (IgG) PFC often differs in our experience (the IgM PFC being less distinct than the IgG PFC) thus necessitating slightly different threshold settings to enumerate these respective antibody

classes accurately. Nevertheless, once a proper threshold is ascertained (which is easily accomplished by visual examination of the flagged PFC on the video monitor), enumeration of PFC on different slides becomes as simple and rapid as it is to place slides into the holder and press the button for the count; the digital readout displays the answer in fractions of a second.

Enumeration of Nucleated Cells in a Hemacytometer.

Suspensions of single spleen cells diluted in 2% acetic acid were placed on a hemacytometer chamber and then examined visually in the light microscope. After counting all cells in the respective corners of the chamber, the television camera was then focused on the chamber and each corner then flagged and counted by the machine. A comparison of visual and instrument counts on 44 different cell suspensions is presented in the scatter diagram shown in Figure 2. As shown here, again a significant correlation between visual and instrument counts was obtained (correlation coefficient = 0.996). As in the case of the PFC slides, it is necessary to adjust the threshold setting to the appropriate level in order to flag all cells appearing in the hemacytometer field. Once this is established, however, performing multiple cell counts becomes a very rapid and accurate procedure by simply visualizing the counting chambers under the television monitor and then pressing the count button which then gives a very accurate enumeration of the flagged nuclei in the field. It is worth noting that in certain cell preparations which contain, inevitably, a certain degree of cell clumping, the threshold settings can be set on the machine such that clumps of this type are not flagged and therefore are not counted by the machine.

DISCUSSION

The Fisher Count-All^{R} is a relatively compact system which, as described here, can be modified to perform semi-automated counting of either plaque-forming cells or nucleated cells in suspension. Since both of these procedures can be accomplished with the same machine, the cost of the unit is quite reasonable considering that the basic unit (excluding the light microscope, which can be quite easily separated from and utilized independently of the machine) is less than the cost of most automatic cell counting systems which are usually limited only to enumeration of particle counts. In addition, since the machine can likewise be utilized for semi-automated counting of radioautographic grains on cell smears, the unit can potentially satisfy three or four major technical needs of modern immunology research laboratories.

Although the unit has only been in use in our laboratory for a short period of time, it is clear that an enormous number of man hours normally spent counting plaque-forming cells visually under the microscope will be spared. As an example, an experiment consisting of 200 PFC slides to analyze with PFC's varying in number from 50 to 500 per slide, would normally require the better part of one person's working day to complete this task. With this machine, this number of slides with PFC's in the range mentioned can be completely counted in approximately 2 hours or less. In addition, and perhaps more importantly, is the fact that alleviation of operator fatigue normally associated with lengthy PFC counting under the microscope, should serve to diminish the extent of human error in making such counts and thereby enhance reproducibility of results.

SUMMARY

A commercially available bacterial colony counter has been modified to permit rapid, highly accurate,semi-automated enumeration of antibody-producing plaque forming cells in semi-solid support medium as well as enumeration of nucleated cells in suspension on a standard hemacytometer chamber. This apparatus should therefore serve as an enormous time-conserving accessory to most modern laboratories involved in immunological research.

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FIGURE LEGENDS

Figure 1.

Correlation of visual and instrument counts of anti-DNP and anti-BPO plaque-forming cells in each of 72 individual PFC slides. For further discussion, see text.

Figure 2.

Correlation of visual and instrument counts of nucleated cells on standard hemacytometer chambers in each of 44 single cell suspensions diluted in 2% acetic acid. For further discussion, see text.

FIGURE 1

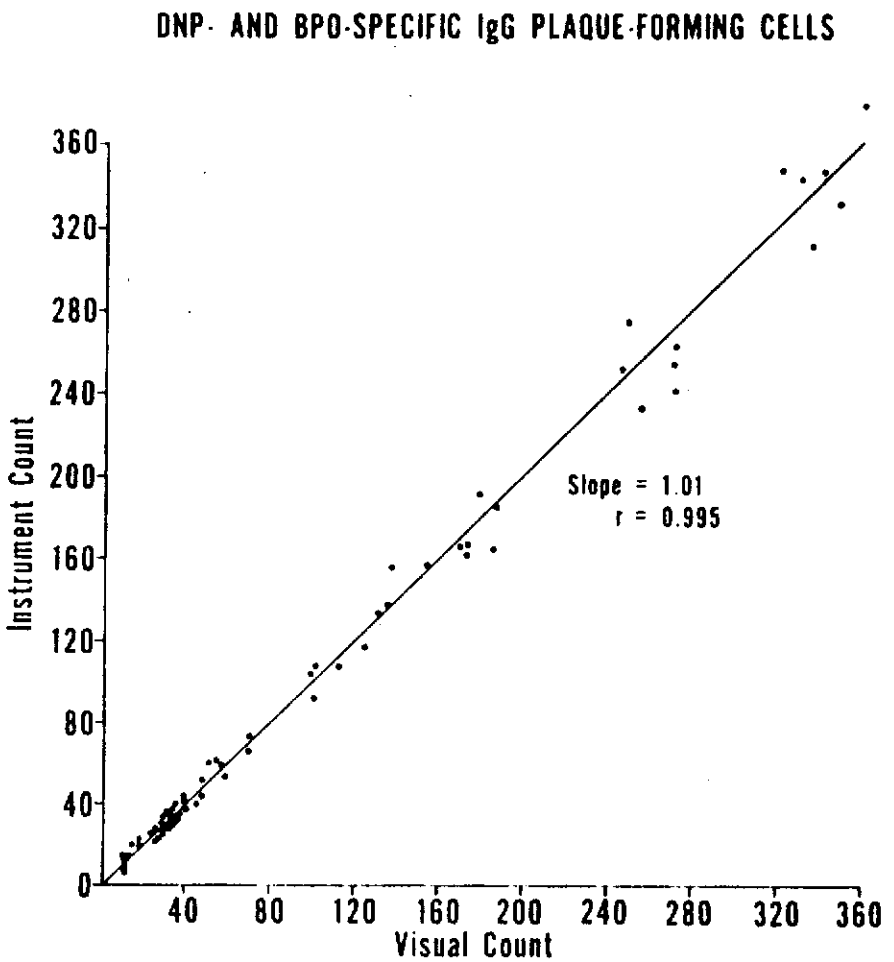
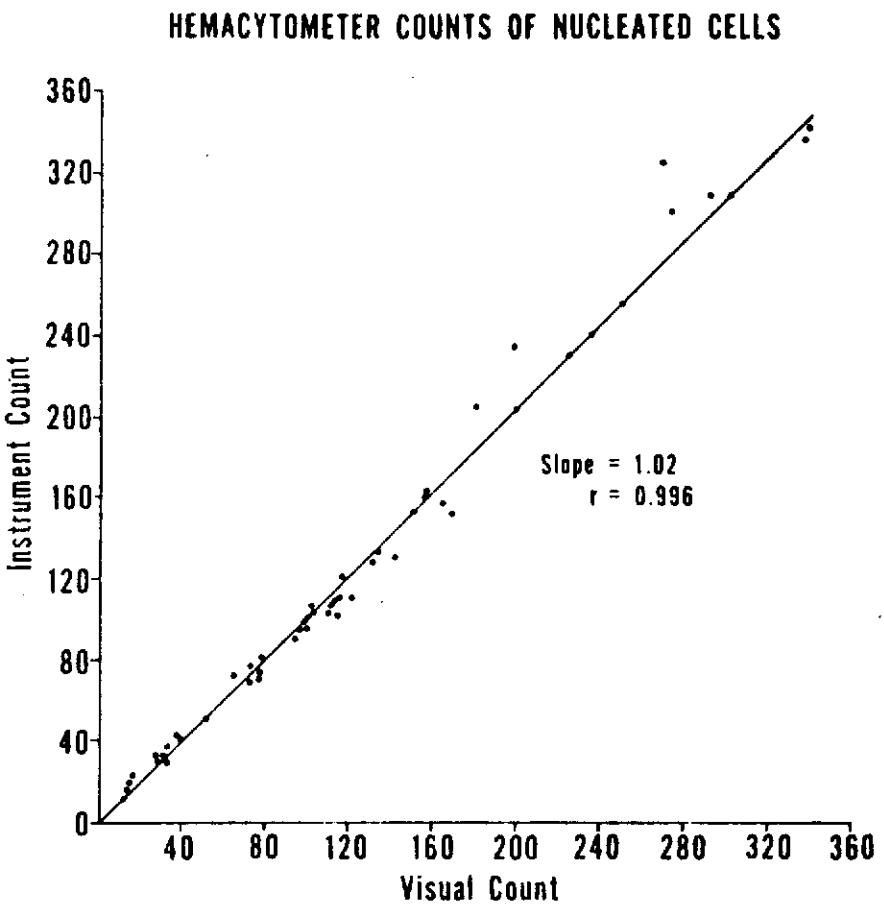

DNP- AND BPO-SPECIFIC IgG PLAQUE-FORMING CELLS
Instrument Count
Visual Count
0
40
80
120
160
200
240
280
320
360
Slope = 1.01
r = 0.995

FIGURE 2


HEMACYTOMETER COUNTS OF NUCLEATED CELLS
Instrument Count
Visual Count
Slope = 1.02
r = 0.996
0
40
80
120
160
200
240
280
320
360

FOOTNOTES

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