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TECHNIQUES

THE APPLICATION OF MICROPHOTOMETRY TO THE INDIRECT IMMUNOFLUORESCENT ANTIBODY TECHNIQUE USING THE HUMAN GASTRIC PARIETAL CELL ANTIGEN-ANTIBODY SYSTEM

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SUMMARY

This paper is an account of our experience in the use of the Leitz microphotometer equipment in the attempt to quantitate the degree of specific fluorescence in the indirect immunofluorescent antibody technique using the human gastric parietal cell antigen–antibody system. The main limitation of the equipment at present is the variability of the HBP 200 W lamp. This fact, together with the biological variation within the tissue section, makes multiple readings necessary. The method is, therefore, time consuming and perhaps mainly applicable to the investigation of standardization of the immunofluorescent technique itself and of the reagents used rather than the quantitation of results obtained in a routine manner using an accepted technique.

INTRODUCTION

Standardization in the immunofluorescent antibody technique can only be achieved if quantitation is used in each stage of the procedure, including microscopy. Microphotometry has been studied by a number of investigators (Mansberg & Kusnetz, 1966; Pittman, *et al.*, 1967; Goldman, 1967) but so far no instrument has been described that is suitable for the ordinary immunology laboratory. A few of the largest manufacturers are now making microphotometry equipment available. The following is an account of our experience in

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attempting to standardize the indirect immunofluorescent antibody technique using microphotometry applied to the parietal cell antigen–antibody system in man with equipment provided by Leitz, Wetzlar, Germany.

MATERIALS AND METHODS

Instrumentation

Microscope

A Leitz Orthoplan large research microscope with a binocular head, phototube and beam-splitter was used. For the present measurements, a fluorite system oil immersion objective Fl 95/1·32–1·10 with iris diaphragm was used in conjunction with a periplan GF × 10 eyepiece in the phototube and dark field oil condenser N.A. 1·20. The viewing eyepieces in the binocular head were periplan GF × 12.

Light source and filters

The light source was a high pressure mercury vapour lamp Osram HBO 200 W fitted in Leitz lamp housing 250 with a reflector and a glass collector. The controls for positioning the lamp, reflector and condenser are placed outside the housing so as to provide rapid and precise centring of the illuminating beam. In addition to a built-in heat absorption filter KG1 2-mm, the lamp housing also included a filter changer which accommodated four transmission filters. These can be inserted in the beam in any desired order by external levers. A neutral diffusing filter together with blue BG12 filters of 5 and/or 3 mm thickness were used for fluorescence excitation. Secondary filter K150 was used for suppression. The microscope was also fitted with a tungsten light source. The object could be examined either under ultraviolet–blue or tungsten light.

Photometer

Microscope photometer MPV with the Knott light measuring device type MFLK, BN 5001 T was used. The light meter consisted of the photomultiplier measuring head type SKV 317 V, photomultiplier type 6094A (S 11 cathode) and the highly stabilized power supply 500–2500 V, 5 mA type NSHM. As shown in Fig. 1, the MPV photometer attachment consisted of a basic body fitted with a viewing telescope, a variable measuring diaphragm (which may be circular or rectangular), housing for a pilot lamp (8 V, 0·3 A) to illuminate the image of the measuring diaphragm, a dividing plate operated by a lever to expose the photomultiplier attachment to light from the object or to enable the image of the measuring diaphragm to be illuminated by the pilot lamp.

The eyepiece (GF × 12) of the viewing telescope had a graticule for reading the aperture of the measuring diaphragm. The aperture of the diaphragm can be adjusted to any position throughout a continuous range from full field down to an area of approximately 0·5 μ^2 or 0·5 μ in diameter.

The Scalamp Galvanometer (Pye, Cambridge, England) served as a measuring instrument. It provided four ranges of sensitivity; full, 1/20, 1/100, 1/1000.

The adjustment of the condenser and objective was carried out in accordance with standard methods of microscopy using tungsten light. The centring of the mercury vapour lamp was checked. The condenser, object slides and coverglasses were thoroughly cleaned. The thick-

ness of the object slides should be between 0·9 and 1·1 mm. For the sake of uniformity, slides of 1·05 mm thickness measured by micrometer were used throughout the present experiment. Coverglass of thickness as near to 0·17 as possible were chosen. Photometer measurements were made in the evenings when powerful electrical equipment in the vicinity was not in use, thus avoiding major fluctuations in the voltage supply which might occur in spite of stabilization.

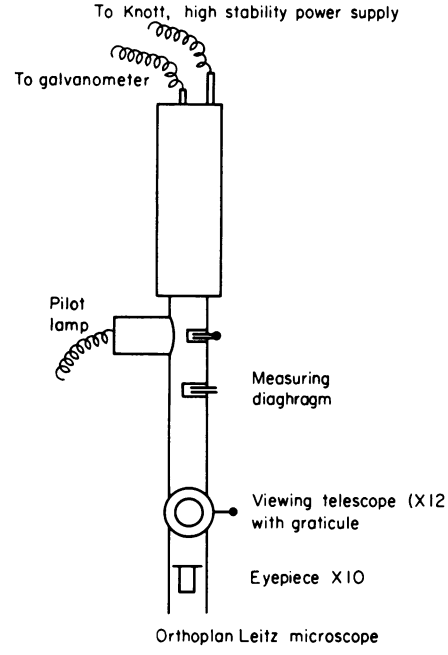

FIG. 1. Diagram of microphotometer attachment.

Fluorescent antibody technique

The indirect fluorescent antibody technique (Coons & Kaplan, 1950) was used.

Sera

*Serum B*2214 was strongly positive for parietal cell antibody and was obtained from a patient with pernicious anaemia.

*Serum B*8805 was negative for gastric parietal cell antibody and was obtained from a control subject. The sera were stored at −20°C. Freezing and thawing were carried out as rapidly as possible.

Tissue

Mucosa from the body of human stomach was obtained from a patient undergoing gastrectomy. The fresh tissue was cut into rectangular blocks of approximately 2–3 mm thickness, placed in small glass vials and rapidly frozen to −70°C in acetone–solid CO_2 mixture. The blocks were stored at −20°C until required for sectioning. Sections were cut as uniformly as possible at 4 μ using a Pearce cryostat at −20°C, air dried by fan for 4 hr,

stored at 4°C and used within 3 days. During storage, the sections were protected in polythene bags from condensation and prior to use the sections were allowed to come to room temperature before being removed from the polythene bag.

Conjugate

Fluorescein in the form of fluorescein isothiocyanate (chromatographically pure isomer I) was conjugated with monovalent antiserum–anti-human IgG in our own laboratory. The horse anti-human IgG was obtained from the Central Blood Transfusion Laboratory, Netherlands Red Cross, Amsterdam. For the purposes of conjugation, the globulin fraction was diluted to approximately 10 mg/ml with carbonate–bicarbonate buffered saline at pH 9. Chromatographically pure isomer I fluorescein isothiocyanate (FITC) was used at a concentration of 1 mg/100 mg protein. The conjugation was done at 4°C for 18 hr. Free fluorescein was removed by filtration through Sephadex G-50 followed by overnight dialysis in veronal buffer, pH 7·2 (Oxoid, England) at 4°C. The fluorescein–protein absorption ratio was 0·67 as determined by Zeiss PMQ II and M4Q III spectrophotometer equipment using wave band 495 mμ for fluorescein and 280 mμ for protein.

The same batch of conjugate (GG4 Lot 11) was used throughout the studies described in this paper.

Immunofluorescent staining

Undiluted and diluted serum, after warming to room temperature, was applied to each section for 20 min at room temperature. The sections were then washed in veronal buffer (pH 7·2) for 30 min with continual gentle agitation on a rolamix machine (Luckham, England), the buffer being changed once during this procedure. The fluorescein–protein conjugate (anti-human IgG–FITC) was applied for 20 min at room temperature. The sections were then given a final wash in veronal buffer for 1 hr with continuous agitation and changing of the buffer every 20 min. The sections were then mounted in 10% glycerol in veronal buffer.

Fluorescent standard

A small qunatity of Zn–Cd sulphide fluor, lot 1023, was obtained through the kindness of Dr Morris Goldman, Department of Immunology, Bionetics Research Laboratories Inc., Falls Church, Virginia. An amount smaller than visible by the naked eye was placed on a clean glass slide. The preparation was then mounted with a coverslip using non-aqueous univert mountant (Gurr, England). After allowing the mounting medium to set firmly, a single crystal was selected as fluorescent standard using the same objective (× 95), condenser and other optics as above. The iris of the objective was always set in the closed position.

RESULTS

Standardization of microscope

The intensity of a new lamp HBO 200 W falls off rapidly within the first few hours of its life and then enters a plateau phase. Using the fluorescent standard, as described above, it was apparent that the intensity of the beam from the HBO 200 W lamp in its plateau phase may remain constant over a period of several hours but that it may suddenly fluctuate by up to 15–20% due to instability of the arc of the lamp.

Assessment of optimal dilution of conjugate

Sections of gastric mucosa were stained in the indirect immunofluorescent technique using positive antiserum B2214 diluted 1:2 in veronal buffer and using serial dilutions of anti-human IgG–FITC conjugate (1:2, 1:4, 1:6, 1:8, 1:10, 1:16 and 1:20). The fluorescence of the cytoplasm was measured in fifty parietal cells in each slide, there being one slide for this purpose for each dilution of the conjugate. The cells were chosen at random throughout the section provided they had an area of cytoplasm large enough to accommodate the square area determined by the measuring diaphragm (10 × 10 on the eyepiece graticule

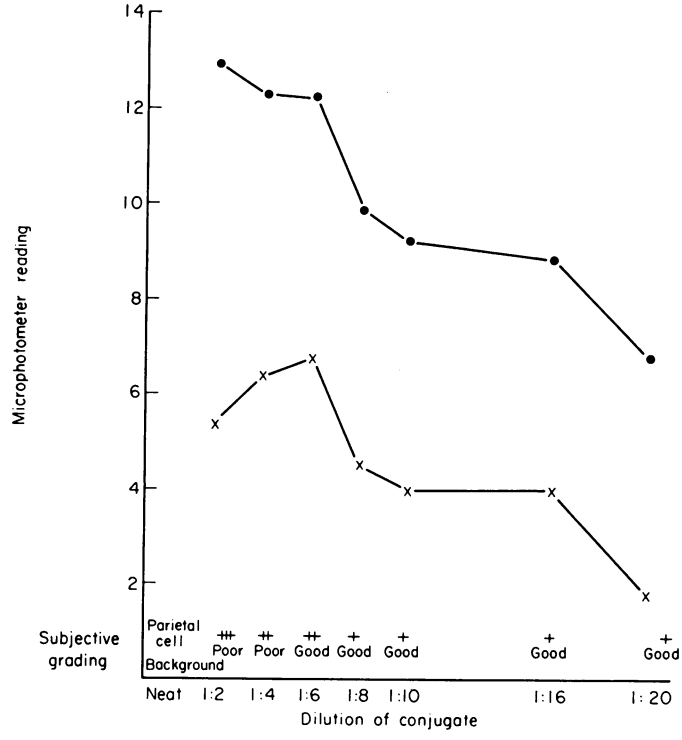

FIG. 2. Determination of optimum dilution of conjugate for use in the indirect immunofluorescent antibody technique. The photometer readings for the contrast between specific (●) and non-specific (×) fluorescence correlate well with the subjective assessment, indicating that a dilution of conjugate at 1:6 is optimum. Positive serum B2214 was used in a dilution of 1:2 throughout. Primary filter 5 mm BG12. The results are shown as the average of measurements on fifty cells for each dilution of conjugate.

of the viewing telescope). Care was taken to ensure sharp focus on the parietal cell cytoplasm and that the area to be measured did not overlap onto the nucleus. This reading was designated as 'specific fluorescence'. The reading from an area of the same size adjacent to each cell was measured in each instance and designated the 'non-specific fluorescence'. The difference between the 'specific' and the 'non-specific' fluorescence is referred to as 'the contrast'. The primary filter in this experiment was Schott 5 mm BG12. The microphotometer readings

were made as quickly as possible (2–3 sec) in order to avoid undue exposure of the tissue section to ultraviolet–blue light. The light beam was switched to tungsten light whenever measurements were not being taken.

Duplicate slides were read subjectively for brightness of parietal cell fluorescence (specific fluorescence) and for brightness of background tissue staining (non-specific fluorescence). The results were correlated with the mean value of the photometer readings for each slide and are shown in Fig. 2. It is seen that the optimum dilution of conjugate to give maximum contrast between specific and non-specific fluorescence was found to be 1:6 both by microphotometry and by subjective assessment.

TABLE 1. Average of micrometer readings of fifty cells in each of ten slides stained with a positive serum and on each of eight slides stained with a negative serum

	Average of fifty readings		
	Specific	Non-specific	Contrast
Undiluted +ve serum B2214			
Slide 1	15	7·15	7·85
2	14	6·20	7·80
3	14·34	6·78	7·56
4	11·74	5·95	5·79
5	11·67	5·78	5·89
6	13·00	6·54	6·46
7	12·10	6·10	6·00
8	12·94	6·50	6·44
9	11·55	5·60	5·95
10	11·47	5·25	6·22
Undiluted −ve serum B8805			
Slide 1	8·55	7·68	0·87
2	7·67	6·78	0·89
3	6·18	5·45	0·73
4	6·48	5·84	0·64
5	6·73	5·94	0·79
6	7·16	6·50	0·66
7	8·88	5·21	0·67
8	5·96	5·23	0·73

Reproducibility of readings from gastric parietal cells showing positive immunofluorescence

Ten slides of gastric sections stained with undiluted positive serum B2214 and eight slides stained with undiluted negative serum B8805 were studied. The conjugate was used in a dilution of 1:6. Measurements were carried out on different days but always within 2 hr of staining.

As in the above experiment, the fluorescence of the cytoplasm was measured in fifty parietal cells in each slide. The measurements were conducted in the same way as above. The findings are shown in Table 1. The standard deviation for the contrast between specific and non-specific fluorescence for the fifty parietal cells measured in slide 10, as an example,

was $\pm 1{\cdot}104$ with a mean of 6·22 and a range of 4·8–9·1. There was no consistent decline in the immunofluorescent readings to indicate that loss of fluorescence had occurred because of exposure to ultraviolet–blue light of those parietal cells that were read towards the end of the series in each slide using intermittent exposure.

Titre of antibody determined by microphotometry and by subjective assessment

Serial doubling dilutions of positive serum B2214 were tested against duplicate slides and microphotometry carried out as described above, the fluorescence of the cytoplasm of fifty

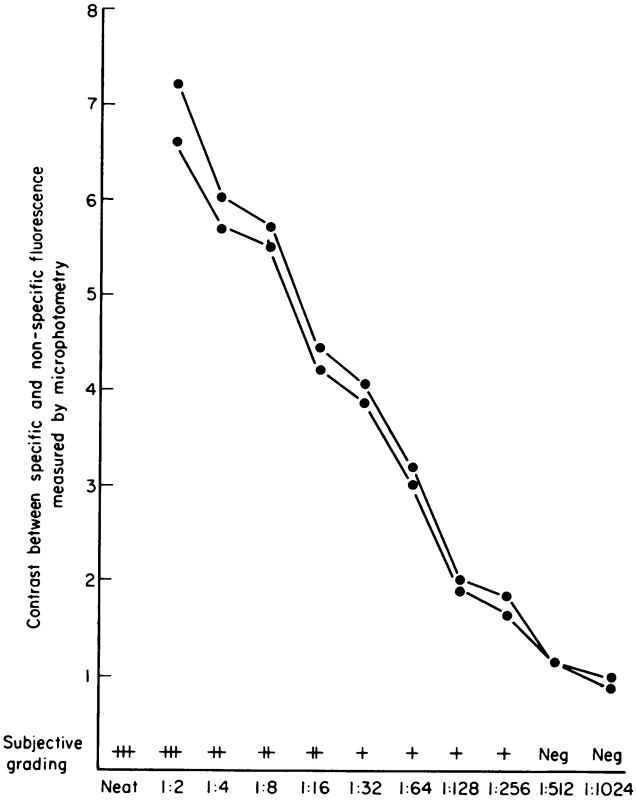

FIG. 3. The correlation between the microphotometry readings and the subjective assessment of possitivity with serial dilutions of positive serum B2214. Measurements were made on duplicate slides for each dilution of the serum. The results are expressed as the mean of the measurements on fifty parietal cells on each slide. Primary filter 5 mm BG12.

parietal cells compared to that of adjacent tissue being measured in each slide. The results of photometry were correlated with the subjective grading of positivity in each of the slides. The anti-human IgG–FITC conjugate was used in a dilution of 1:6. Duplicate slides were always examined with the same microphotometer settings and on the same day (1·4 kV, sensitivity 1/20, primary filter 5 mm BG12). The results are shown in Fig. 3.

Photodecomposition of fluorescein conjugate on exposure to ultraviolet–blue light

Fig. 4 shows the fall-off in intensity of fluorescence in the gastric parietal cell antigen–antibody system on continuous exposure of individual cells to ultraviolet–blue light using primary filter 5 mm BG12 and primary filter 5+3 mm BG12. It is clear that the fall-off in immunofluorescence is more rapid with the 5 mm BG12 filter as compared to the 5+3 mm BG12 filter although the intensity of fluorescence is much higher, especially initially with only the 5-mm filter. However, a greater contrast between the specific staining and the non-specific staining is obtained for a longer period with the 5+3 mm BG12 filters.

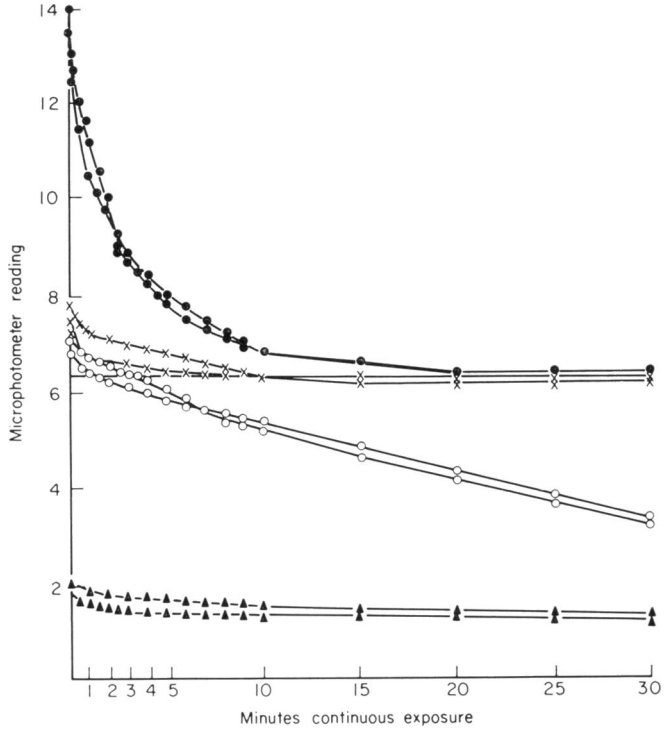

FIG. 4. The microphotometer readings of the cytoplasm of gastric parietal cells stained in the indirect immunofluorescent antibody technique (specific fluorescence) and of the adjacent gastric tissue (non-specific fluorescence). The readings were taken during continuous exposure to ultraviolet–blue light using primary filters 5 mm BG12 and 5+3 mm BG12, respectively. ●, Specific (primary filter 5 mm BG12); ×, non-specific (primary filter 5 mm BG12); ○, specific (primary filter 5+3 mm BG12); ▲, non-specific (primary filter 5+3 mm BG12).

At this juncture, a new mercury HBO 200 W lamp was introduced giving a much greater intensity of illumination than the previous lamp that had been used for many hours. The setting of the photometer had to be altered entirely with regard to voltage sensitivity. Fig. 5 shows the effect of continuous exposure on specific staining to excitation using a 5+3 mm BG12 blue primary filter compared to the effect of intermittent exposure when the beam

was switched to tungsten light between the readings, each of which lasted from 2 to 3 sec. Fig. 5 shows that the greater the initial intensity of the fluorescence the more rapid is the photodecomposition, as illustrated by curves 1–4 which are consecutive during the early hours of the lamp's life. The greater intensity of illumination produced by excitation with 5 mm BG12 filter caused more rapid photodecomposition of fluorescence than a weaker intensity of illumination produced by 5+3 mm BG12. Intermittent exposure by radiation produced by the combination of 5+3 mm BG12 gave very little fading of fluorescence and this filter arrangement was, therefore, used for the following experiment.

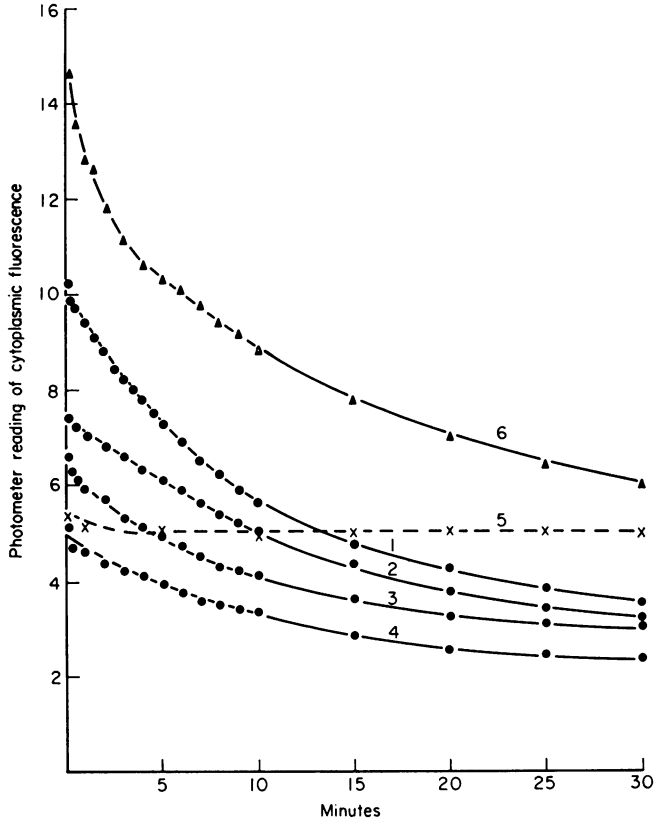

FIG. 5. Photometer readings from the cytoplasm of gastric parietal cells stained in the indirect immunofluorescent antibody technique with serum B2214. Curves 1–6 are consecutive. The cells were subjected to continuous exposure to ultraviolet–blue light except in experiment 5, when the exposure was intermittent (approximately 2 sec for each measurement). Primary filters as shown. ▲, 5 mm BG12, continuous exposure; ×, 5+3 mm BG12, intermittent exposure; ●, 5+3 mm BG12, continuous exposure.

Comparison of intensity of immunofluorescence of gastric parietal cells in the upper and lower part of the gastric crypts

Four sections of gastric mucosa were stained with neat positive serum B2214 and the anti-IgG–FITC conjugate in a dilution of 1:6. Forty parietal cells were measured for each

slide, twenty chosen from cells in the region of the mucous neck glands and twenty chosen from deep in the gastric crypts. The cells were selected from the superficial and deep parts of the gastric sections alternately using tungsten light. The exposure of the gastric sections to ultraviolet–blue illumination was intermittent and for the measurement of fluorescence only. Primary filters 5+3 mm BG12 were used. The aperture of the measuring diaphragm was 10 × 10 as throughout the whole of the series of experiments. The results of this study are shown in Fig. 6. It is clear that the intensity of fluorescence of the deeper cells is statistically greater than is the immunofluorescence intensity of the more superficial gastric parietal cells. This difference is apparent subjectively (see Fig. 1 of Irvine, 1963).

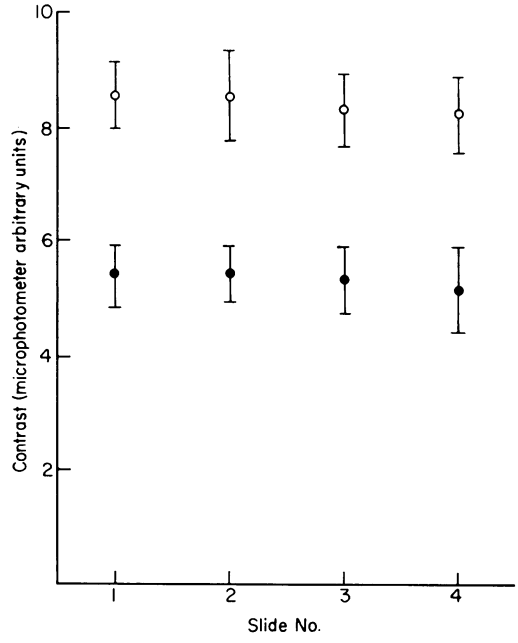

FIG. 6. Illustrates the difference in the intensity in positive immunofluorescence (specific minus non-specific) in the cytoplasm of human gastric parietal cells in the upper (●) and in the deeper (○) part of the crypts. Four sections were stained by the indirect immunofluorescent method with serum B2214. Twenty superficial and twenty cells deep in the gastric mucosa were measured with the microphotometer. The results are shown as the mean values ±1 SD.

DISCUSSION

One inherent defect in the present system is the instability of the arc of the HBO 200 W lamp. This may be overcome by substituting an HBO 100 lamp. The stability of the arc of this latter lamp would merit the provision of a highly stabilized voltage unit for that lamp.

The variation in stability of the HBO 200 W lamps in their plateau phase was estimated to be 15–20% in the present study and the oscillation within that range was random. For periods of variable length, the lamp could be quite stable (±1%) and then oscillate. The fluctuation in the intensity of the light source would, therefore, be compensated for to some

extent by taking multiple readings and determining the mean value with standard deviations where necessary.

Biological as well as instrumental variability must also affect the degree of reproducibility of the photometer readings. Even if the perfect tissue section could be cut so as to be of uniform thickness throughout, the cytoplasm of different parietal cells would be cut at varying angles so that different thicknesses of fluorescing material may be available for different cells. As shown in Fig. 6 of the present paper, parietal cells situated in different parts of the gastric section vary in their intensity of fluorescence in the fluorescent antibody technique. Cryostat sections are not perfect sections. They are likely to vary in average thickness and in thickness within each section. This must affect the amount of fluorescence emitted, particularly when transmitted rather than incident light is used. Again, as with the light source, the variation in these factors would be random or can be randomized. Hence, remarkably reproducible results may be achieved (see Figs. 2 and 4) when the mean readings from fifty cells are plotted for duplicate slides with serial dilutions of antiserum.

In spite of the extreme sensitivity of the microphotometer, it is of interest that, at least with regard to the gastric parietal cell antigen–antibody system, the findings with microphotometry correlate very closely with the subjective interpretation. This was particularly clear in the titration of an antiserum.

The main application of microphotometry in immunofluorescence work at the present time would appear to be in defining the procedure of preparing immunofluorescence specimens to give optimum results. Microphotometry should be useful in determining the best buffer to use as diluent, the optimum dilution of conjugate and its staining characteristics (Pittman *et al.*, 1967) in comparison to a standard conjugate, washing times for sections to give optimal contrast between specific and non-specific fluorescence, the most suitable mounting medium for fluorescence preparations, the most appropriate primary and secondary filters and the choice of other optics to give maximum contrast and minimum background fluorescence and as a check on the performance of the lamp.

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