the plates were covered with Dulbecco phosphate-buffered saline (for direct, 19S producing plaques), or with species specific anti-γ-globulin antiserum (for indirect, 7S producing plaques)[19,20]. After an additional 90 min incubation, the plates were rinsed and covered with 1 ml. of reconstituted, lyophilized guinea-pig complement (Difco) diluted 1 : 10 in Dulbecco phosphate-buffered saline. Haemolytic plaques developed during 2 h of incubation with complement at 37° C, and were counted without magnification in direct light by two individuals.

Table 2. APPEARANCE OF ANTI-DNP FORMING CELLS IN MICE FOLLOWING PRIMARY INJECTION OF DNP-RABBIT SERUM ALBUMIN*

Day	PFC/10^7 spleen cells† Direct	Indirect‡
0	3	3
3	14	3
7	700	420
11	161	462

* Balb/c mice immunized by intraperitoneal injection of 0·4 mg DNP-rabbit serum albumin in 0·2 ml. of complete Freund's adjuvant.

† Average of three animals.

‡ Developed with goat anti-mouse IgG (Hyland Lab. lot GP 7–66) diluted 1 : 50.

This technique was applied to the assay of cells forming antibodies against two different haptens and one protein in mice, rabbits and guinea-pigs. The results (Tables 2 and 3) show rapid increase of "direct" PFC following the third day after immunization and an increase in "indirect" PFC occurring later, similar to those reported by others[3–9]. They also demonstrate that by this technique a very low background is observed and PFC of hapten (DNP) or carrier (BSA) specificity can be determined simultaneously (Table 3). The plaques detected with DNP-Fab–RBC were completely inhibited by DNP-amino caproate (10^{-5} M) and those detected with BSA-Fab–RBC were inhibited by BSA (5×10^{-7} M). In the case of rabbit spleen cells it is especially important to have highly specific anti-Fc antiserum for the elucidation of indirect PFC, otherwise non-specific lysis of the Fab coated RBC will occur. Similarly it is also not advisable to use IgG as a carrier or immunogen, for a cross-reaction of anti-IgG and the rabbit Fab used to coat the cells might confuse the results (see Table 3).

Table 3. ANTIBODY FORMING CELLS IN RABBIT SPLEEN AT DIFFERENT STAGES AFTER IMMUNIZATION*

Immunogen	Day	Erythrocytes coated with	PFC/10^7 spleen cells Direct	Indirect†
DNP-BSA	5	Fab	20	25
		DNP-Fab	70	30
		BSA-Fab	80	40
DNP-BSA	Hyperimmune	Fab	20	30
		DNP-Fab	40	13,000
		BSA-Fab	30	1,480
PEN-bovine IgG	13	Fab	37	39
		PEN-Fab	34	98

* Immunization was by multiple intradermal injections of 2 mg immunogen in complete Freund's adjuvant.

† Developed with goat anti-rabbit-Fc previously adsorbed on rabbit-Fab–'Sepharose' column.

Our technique has the following advantages: a series of modified anti-RBC-Fab preparations can be prepared and used as reagents for the assay of PFC with specificity towards the attached ligand. These reagents can be titrated, stored and subsequently used many times with high reproducibility for the coating of erythrocytes. The assay is applicable to both small and large molecules and can measure PFC towards either hapten or carrier in the same system, although the methods and optimal conditions for attachment of the required moiety should be investigated in each case. The attachment of haptens seems quite straightforward in many cases and the effect of hapten density on plaque formation can be studied by varying the number of ligands on the Fab or the number of modified Fab per RBC. The binding of proteins presents more difficulty and the use of bifunctional reagents other than glutaraldehyde is now under investigation in order to improve the assay for protein antigens. The coating of the RBC by the modified Fab is fast and simple and does not change the stability of the RBC. The background PFC is very low and the assay of different systems can be compared simultaneously, for all of them have the anti-RBC-Fab as a common denominator.

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Application of Electron Probe Microanalysis and Electron Microscopy to the Transfer of Antigenic Material

We have studied the effect of various antilymphocyte sera on lymphocytes, using the Stereoscan electron microscope[1], and more recently we described the formation of long intercellular processes between macrophages and lymphocytes in certain conditions[2]. Process formation was particularly marked if macrophages from rats sensitized to tuberculin were incubated first with PPD and then with either normal lymph node cells or lymph node cells from rats sensitized to tuberculin (Fig. 1). In the former case, many macrophages were seen to be closely surrounded by clusters of lymphocytes.

We describe here an attempt to produce evidence for the hypothesis that transfer of antigenic material from cell to cell occurs along these intercellular processes. The antigenic protein used was PPD. It was labelled with iodine and subsequent cell preparations scanned for iodine distribution by X-ray microanalysis (EMMA-4, AEI Scientific Apparatus Ltd). Previous studies of biological material using this technique have been reviewed by Robertson[3], while methods of specimen preparation have been discussed by Andersen[4], and Beaman *et al.*[5]

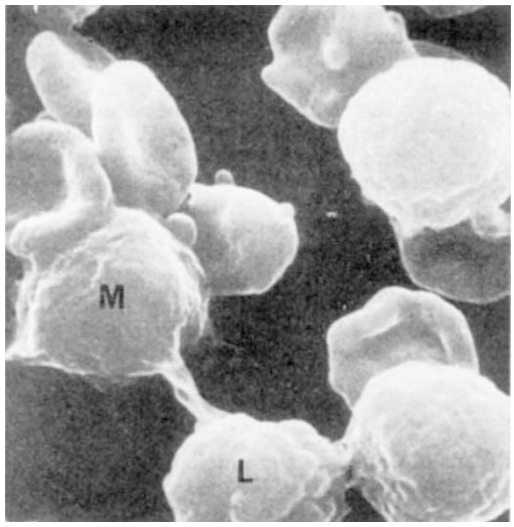

Fig. 1. Scanning electron micrograph of a typical intercellular process between a lymphocyte (L) and a macrophage (M). Some distorted red cells (due to incubation in the experimental conditions) and two other lymphocytes can also be seen. (×2,250.)

have investigated the titanium and zinc content of blood cells.

Two mg of PPD was dialysed for 24 h against 0·15 M phosphate buffer at *p*H 7·2; 0·05 ml. of 0·1 N iodine in 0·15 per cent potassium iodide was then mixed with the PPD. 0·2 ml. of a 'Chloramine *T*' solution containing 500 μg of the substance was added drop by drop to the mixture after PPD and iodine and the whole mixed for 10 min. 1·0 mg of sodium metabisulphite was then added. The final mixture was drawn through two small quantities of 'Dowex' resin and sterilized by filtration through 'Millipore' filters (pore diameter 0·45 μm).

Rats were sensitized to tuberculin with Freund's complete adjuvant (CFA-Difco) in a dose of 0·5 ml. per animal. In each case, 0·1 ml. was injected into the superficial cervical lymph nodes, 0·1 ml. into each of the hind foot pads and 0·2 ml. into the back of the neck. Four weeks later, sensitized macrophages were harvested by peritoneal washing. 5×10^7 macrophages in 1·0 ml. were incubated for 30 min at 37° C with 1·0 μg labelled PPD. The macrophages were then washed four times in medium 199 and incubated with the same number of sensitized rat lymph node cells suspended in medium 199 for 30 min at 37° C.

The cell suspension was washed in isotonic phosphate buffer *p*H 7·4 and fixed for 1 h in isotonic 0·5 per cent glutaraldehyde in phosphate buffer. The fixed cells were washed in distilled water and the cell suspension transferred to an electron microscope grid coated with carbon. The cells were dried by the "critical point" method[6].

Intercellular processes were numerous and easily seen (by direct transmission imaging in EMMA-4) (Fig. 2). The processes varied between 2 and 30 μm in length and between 0·3 and 4·0 μm in diameter. Particular features of the specimen (Fig. 2) were chosen and the illumination focused onto these areas by exciting the "mini-lens" of the microscope to produce a probe of approximately 1000 Å in diameter. The area for analysis was thus exactly located. X-rays emitted from the specimen were passed into the spectrometer and tuned for particular wavelengths by adjustment of the spectrometer geometry. X-rays detected in the spectrometers were counted for a selected time at each area of the specimen. The count recorded was corrected by subtracting the background reading (with the spectrometer off the characteristic wavelength of iodine) from the peak reading (with the spectrometer tuned on the characteristic wavelength). As a check that the focused probe was always on the selected area during analysis, the high phosphorus count entering a second spectrometer was observed—this also gave some indication of the mass thickness of the material.

With the probe over individual cells, corrected counts for iodine varied between 74 and 497 per 10 s. Every cell examined contained iodine, so it seemed reasonable to assume that, in a mixture containing equal numbers of macrophages and lymphocytes, iodine labelled PPD must have been transferred to the lymphocytes from macrophages. The cell marked *A* in Fig. 2, over which a count of 74 was obtained, was used to check the spectrum of iodine. Fig. 3 shows that the true presence of iodine at that point was confirmed.

When the intercellular processes were examined, lower but still significant counts were obtained. For example, the corrected counts for iodine over a period of 10 s for the points along the process shown in Fig. 2 were as follows: *B*, 91; *C*, 4; *D*, 11; *E*, 4; *F*, 95. This characteristic of a higher count at the centre of a process than on each side of the centre was found on several occasions. With other processes, there was a steady fall in iodine counts with increasing distance from a cell, reaching a minimum at the centre of the process and rising again as the second cell was approached. In some instances, no iodine was detected in processes extending from cells which had not yet formed intercellular processes.

The error involved in any counts made on the specimen was given statistically by the root mean square of the count. In particular, where low counts were detected, these values were checked for accuracy a number of times.

Other possible sources of error lay in the liberation of iodine from the iodo-protein. In these circumstances one would have expected to detect iodine lying outside cells or intercellular processes. This, in fact, never occurred. Iodo-proteins are known to be reasonably stable in the experimental conditions used. It was therefore concluded that any iodine count recorded originated in protein-bound iodine.

The potentiality of the EMMA-4 analytical electron microscope in detecting small quantities of elements at a cellular or intracellular level has thus been demonstrated. The measurements in this instance were of a qualitative nature, but quantitative estimates are possible. The results suggest that iodine attached to PPD can pass along intercellular processes from cell to cell. The instru-

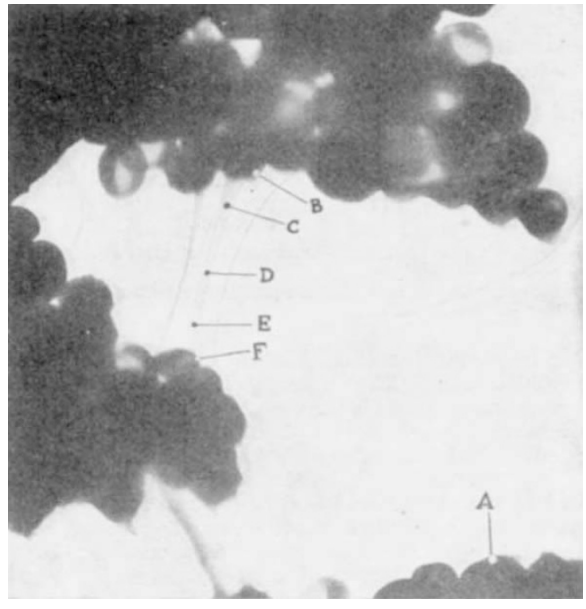

Fig. 2. Transmission electron micrograph (EMMA-4 at 100 kV) of intact groups of cells and intercellular processes. Letters indicate points of analysis for iodine mentioned in the text. (×625.)

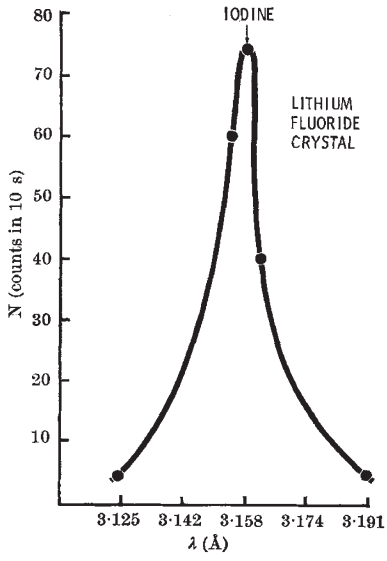

Fig. 3. Graph of counts at various wavelengths over point *A* to confirm the true presence of iodine.

ment can detect sodium and heavier elements. Failure by other analysing techniques accurately to locate elements has previously made this kind of investigation impossible.

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Synergism between Thymocytes and Bone Marrow Cells in a Graft versus Host Reaction

We wish to describe synergism between two distinct populations of cells in the production of cellular immunity. Much work has concerned these cell types, particularly in relation to humoral immunity, and we shall first distinguish them. Both cell populations originate in the bone marrow. One type migrates to the thymus where it acquires special properties[1,2]; while there it is referred to as a thymocyte, but it may join the peripheral circulation and is then called a thymus-derived lymphocyte (TDL)[3,4]. The other type, the bone marrow-derived cell (BMDC), leaves the marrow to populate lymphoid organs or join the circulation, but does not pass through the thymus[1–4].

In 1966 Claman, Chaperon and Triplett reported synergism between thymocytes and bone marrow cells in the production of humoral immunity[5]. Soon afterwards Davies *et al.* showed that while TDL did not produce antibody themselves, they greatly increased the ability of BMDC to do so[6]. Davies and co-workers, and Mitchell and Miller, have further defined the roles of these cells[7,8]. The question naturally arises whether a similar synergism exists in cellular immunity, which is known to be highly thymus dependent[9,10]. Simonsen's work on graft versus host (GVH) disease showed that the splenomegaly which occurs in F1 hosts is caused by cellular immunity, and provides a reliable measure for the GVH reaction[11]. We therefore tested the ability of thymocytes and BMDC to act synergistically in producing cellular immunity by using the GVH reaction as an assay in the manner of Simonsen.

Six week old, sublethally irradiated (C3H/HeJ × DBA/2J) F1 mice (CDF mice) were divided into several groups, each of which received intravenous injections of thymocytes, BMDC, spleen cells, or a combination of thymocytes and BMDC. A total of 3×10^7 cells per mouse was always given, half that number being contributed by each cell type in combinations. Experimental and control cells came from C3H/HeJ and CDF mice respectively. C3H and DBA mice differ at the *H2* locus (*H2k* and *H2d*), causing reliable GVH reactions[11].

To find the time at which the GVH reaction was most prominent the CDF hosts were killed 6, 8, 10, 12 and 14 days after inoculation. Their spleens were removed, weighed, and spleen-to-body weight ratios determined. Each experiment was repeated at least once. Some 260 mice were studied, and results were statistically analysed with Student's *t* test. Histological sections were taken of all spleens. Qualitative evidence of GVH disease was considered convincing when there was destruction of Malpighian follicles, infiltration of their regions by large pyroninophilic cells, and extensive proliferation of cells in the red pulp[11].

The CDF hosts received 350 rad of total body irradiation 12 h before inoculation. This sublethal dose has been shown to enhance the susceptibility of adult mice to GVH disease by causing lymphoid depletion, which seems to make more room for donor cells to contact host tissue[12,13].

To obtain spleen cells, fresh, adult spleens were cut into thirds, squeezed between glass slides, filtered, centrifuged, washed, and suspended in 'Medium 199' under sterile conditions. Viable cell counts were done with trypan blue. To obtain thymocytes, fresh weanling thymuses were cut in half and prepared like the spleen cells. For bone marrow-derived cells, six week old mice were thymectomized and lethally irradiated one week later with 850 rad, immediately following which they were given 5×10^6 bone marrow cells intravenously from syngeneic donors. Thirty days later their spleens were collected and cell suspensions were made as above. The combinations of thymocytes and BMDC were mixed *in vitro* and given at the same time as the other cells.

Parental spleen cells caused the most consistent and severe GVH reaction (Fig. 1). It was most prominent after 12 and 14 days, although it first appeared on the tenth day. None of the cell types caused any apparent GVH reaction before 10 days. C3H/HeJ thymocytes did not cause splenomegaly as compared with CDF thymocytes or radiation controls (Fig. 1); rather their spleens were significantly smaller than those controls ($P < 0{\cdot}001$). C3H/HeJ BMDC produced no splenomegaly as compared with CDF spleen cells (*P* not significant) after 14 days (Fig. 1). In one experiment, however, they were larger than radiation controls after 10 days, but were slightly smaller after 14 days. At no time did they produce any histological evidence of GVH reaction, so that we conclude that BMDC alone did not cause GVH reaction in these doses. Parental thymocytes and BMDC combined *in vitro*