rat is situated at a topologically similar locus as the presently found area.

Future research will have to show whether the zone of the cat's cortex described can also be termed prefrontal on the basis of its functional characteristics.

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Changes in the plasma membrane surface of lymphocytes stimulated in vivo with DNCB

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Summary. Stereological principles have been used to evaluate ultrastructural changes which accompany the transformation of lymphocytes stimulated in vivo with dinitrochlorobenzene (DNCB). Whereas unstimulated lymphocytes and blast cells have slightly more than the minimal containing plasmalemmal surface for their volume, stimulated (blast-derived) lymphocytes have an excess surface area in the order of 30%. This observation is discussed in the context of altered cell function and the biosynthesis of additional membrane components.

During cell-mediated immune responses, T-lymphocytes in the thymus-dependent paracortices of draining lymph nodes are stimulated to transform into blast cells which then divide to produce a new generation of lymphocytes[2,3]. These daughter lymphocytes are functionally well-defined and are classified as killer, helper, suppressor or memory cells according to their subsequent activity[4,5]. Recently, Dunlap et al.[6] have demonstrated biochemical differences between the plasmalemmae of unstimulated and stimulated lymphocytes. However, little or no difference between functionally distinct populations of lymphocytes is apparent on purely morphological inspection.

In this report, using conventional stereological techniques[7] we establish ultrastructural differences between unstimulated lymphocytes and cells stimulated in vivo with DNCB. In particular, there is a marked increase in the plasma membrane surface area of stimulated lymphocytes. The quantitative morphological changes probably reflect the altered functional status of stimulated cells.

Materials and methods. Adult male C57 Black mice were sensitized by painting 10% DNCB in acetone on to their shaved right flanks. 4 days after sensitization, when blast cells are maximal in the draining axillary lymph nodes[8], 1 group of mice was killed and their axillary nodes removed and processed for electron microscopy. Each mouse of a second group was injected with 1 µC of tritiated thymidine per g b.wt at 4 days after sensitization and sacrificed at 6 days. At this time after labelling, there is a maximal number of labelled (daughter, stimulated) lymphocytes in the nodes. A third group of mice was untreated; their nodes were removed to provide a source of unstimulated lymphocytes.

All tissue was processed in a standardized fashion[9]. Thin strips of lymph node tissue were fixed in 3% buffered glutaraldehyde at pH 7.3, postfixed in 2% aqueous osmium tetroxide and embedded in Araldite resin after dehydration. A Huxley (Cambridge) ultramicrotome was used for sectioning. Semithin (ca. 0.5 µm) sections were cut for light microscopic measurements and ultrathin (ca. 70 nm) sections for electron microscopy. Autoradiographic preparations were coated with Ilford L4 nuclear emulsion, exposed for 20 weeks and developed in Kodak D19 developer. Stimulated cells were identified as those lymphocytes having silver grains overlying the nucleus.

From toluidine blue-stained sections, light micrographs of paracortical areas were sampled randomly and printed at a final magnification of $\times 2450$. These prints were used for estimating mean nuclear volumes of unstimulated lymphocytes, immunoblasts and stimulated cells. About 112 micrographs were taken to represent each node. Nuclear profile sizes were evaluated by measuring their long (a) and short (b) axes. Each profile was then converted to an equivalent circle of diameter $d=(a \cdot b)^{1/2}$. Equivalent diameter histograms were corrected by the Schwartz-Saltikov regime[10] to obtain estimates of mean nuclear diameter. As a convenient approximation, mean nuclear volumes were computed for spheres of this diameter.

A systematic sampling of cell profiles was performed using an AEI-Corinth electron microscope. To ensure unambiguous recognition of cell types, only cell profiles containing a nucleus were recorded[11]. Final print magnification was $\times 19{,}650$ as determined from micrographs of a grating replica. Morphometric data were recorded with the aid of a coherent double lattice test system superposed on to each of

Morphometric differences between unstimulated lymphocytes, immunoblasts and stimulated lymphocytes

Parameter	Unstimulated	Immunoblast	Stimulated
Nuclear diameter (μm)	4.25	6.80	4.95
Nuclear volume (μm³)	40	165	63
Nuclear volume density (%)	59	42	56
Cell volume (μm³)	69	393	114
Cell surface-to-volume ratio (μm²/μm³)	1.29	0.72	1.31
Cell surface area (μm²)	89	283	150
Equivalent sphere surface (μm²)	82	260	114
Surface amplification factor	1.09	1.09	1.31

Values are the overall means of 2 experiments.

112 micrographs. Minimal sample size requirements were assessed by cumulative mean plots[12].

Standard stereological relationships were invoked to estimate morphometric parameters of interest. The fraction of cell volume occupied by the nucleus was estimated by point counting and the values corrected for the effects of nuclear-biased sampling[13]. Mean cell volumes were calculated from nuclear volumes and volume densities. Cell surface-to-volume ratios were determined using the method described by Chalkley et al.[14]; combined with cell volume information, these data allowed us to calculate cell plasma membrane surface areas. Finally, a measure of how much more surface area each average cell had than an equivalent sphere was determined by comparing the surface area of each with that of a sphere of equivalent volume. The ratio provided an index of membrane surface amplification, useful for comparing cell types.

Results and discussion. The results of our morphometric evaluations of cell volumes and surfaces are summarized in the table, the values representing overall means for 2 experiments. Unstimulated lymphocytes possess a mean cell volume of about 69 μm³ and a plasma membrane surface of some 89 μm². This surface is therefore approximately 9% greater than that of an equivalent sphere. The average immunoblast has almost 6 times the volume of the average unstimulated lymphocyte and has over 3 times the membrane surface area. This surface again is about 9% greater than that of an equivalent sphere.

In contrast to unstimulated lymphocytes, labelled cells (the progeny of dividing immunoblasts) are larger and have more surface membrane. Moreover, the increase in cell surface is in the same proportion as the increase in cell volume so that the average stimulated lymphocyte has a surface considerably greater (31%) than its equivalent sphere. This means that stimulated cells tend to have a more irregular contour than unstimulated lymphocytes and immunoblasts. Comparison with other published morphometric data is not possible because earlier studies were primarily concerned with relative and absolute volumes of cells and their organelles and not with the cell surface[13,15,16].

It has been shown recently that the plasmalemma of unstimulated lymphocytes has 3 distinct protein components which are also possessed by immunoblasts and by stimulated lymphocytes. Moreover, the membrane of stimulated cells has an *additional* component which is not found in the precursor membranes. It is tempting to speculate on the possible correlation between these structural and biochemical data, on the functional significance of the increased membrane biosynthesis and on the possible roles of the additional membrane components.

The transformation of unstimulated T-lymphocytes into immunoblasts is marked by a 3-fold increase in mean cell surface area. Clearly, a major aspect of this phase of cell transformation is a considerable nett synthesis of plasmalemma which occurs despite a relatively poor development of Golgi elements and rough endoplasmic reticulum[17]. The present results show that the production of stimulated cells from dividing blast cells must also be accompanied by a period of membrane synthesis.

Our autoradiographic studies[18] suggest that at least 4 stimulated cells arise from each blast cell. If the cell surface membrane on the average blast cell (283 μm²) is apportioned equally to each daughter cell - without any additional membrane synthesis - then only about 71 μm² of membrane is available to each. However, a stimulated cell of volume 114 μm³ needs a *minimum* cell surface of 114 μm² which represents a total of 455 μm² for 4 daughter cells or about 61% more than the membrane surface investing the average immunoblast. In fact, 4 stimulated cells have a combined surface of 600 μm² (4× 150 μm²) which is 112% more than that available. Therefore, the dividing immunoblast effectively has to *double* its cell surface area in order to meet the needs of its daughters. These levels of nett membrane synthesis offer adequate scope for the manufacture and incorporation of the new components found by Dunlap et al.[6].

Immunoblast morphology indicates a cell engaged in a high level of protein synthetic activity: it has a well-developed Golgi complex and rough endoplasmic reticulum, large numbers of polyribosomes and a large nucleus containing a prominent nucleolus[9,17]. The nucleus has relatively dispersed and poorly staining euchromatin and this state of chromatin is also associated with a high degree of synthetic activity[19] and may be indicative of gene derepression[20] during blast transformation.

On the functional classification proposed by Bach et al.[4], it seems likely that the majority of cells generated by blast transformation are proliferating helper T-lymphocytes[6] although the presence of other types cannot be discounted. Without a functional definition of cell type it is difficult to be too specific about the significance of a large plasmalemma surface or of new membrane components.

A particular membrane component could be important in the recognition of antigen during graft destruction or it could carry 'memory'. The significance of additional membrane is less easy to interpret. For killer, helper or suppressor cells a larger surface area might facilitate the making of cell-to-cell contacts but additional plasma membrane on memory cells would seem superfluous.

The true significance of the biochemical and ultrastructural changes which accompany the production of stimulated lymphocytes will become clearer when the different (functional) subpopulations affected by these changes can be defined with more certainty. In this study at least, stimulated lymphocytes are larger and have more surface membrane than is necessary merely to contain their volume.

This surface seems to show some unique biochemical characteristics which may be of functional significance.
More recently we have studied human lymphocytes stimulated in vitro with phytohaemagglutinin[9]. Again there is a considerable nett synthesis of plasma membrane: after 3 days, the average blast cell has more than 3 times the surface of the unstimulated lymphocyte. Unfortunately, we do not have any morphometric data on the characteristics of daughter lymphocytes in this model.

1 We wish to thank Prof. R. Barer for his continued support and encouragement. The work was undertaken by M.M. A-H. in part fulfilment of requirements for the degree of Ph. D. in this department. M.M. A-H. was supported by the Ministry of Higher Education and Scientific Research, Government of Iraq.

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Inhibition of fading in fluorescence microscopy of fixed cells

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Summary. Following Hirschfeld et al., dithionite was added to mounting media to inhibit fluorescence fading. Excellent response is reported for fluorescein, acridine orange, 33258 Hoechst, acriflavine, berberine (and ethidium bromide), but not for quinacrine.

The sensitivity advantage of fluorescence techniques is severely diminished by photochemical fading of the fluorescence, a process accelerated by intense excitation[2-6]. Fortunately, the once remote hope of slowing these processes by mounting the sample with a chemical fade retardant has been fulfilled[7]. The prescription of Hirschfeld et al.[7] is to mount cells stained with ethidium bromide in 0.01 M phosphate buffer at pH 7.2, containing 0.02 M sodium dithionite ($Na_2S_2O_4$).
Here, the effect of dithionite was tested on several other fluorochromes of importance. As the sample material was irrelevant to the case, we chose the inner cuticles from mature bulbs of onion. Only fixed samples were tested, since application to live cells involved the problem of their tolerance to 0.02 M dithionite. A compromise suitable for live cells or their constituents is not excluded, though.
The samples were fixed for 15 min in 3:1 ethanol-acetic acid, then stored in cold ethanol. After being brought to water in a graded ethanol series, the samples were stained for 10 min in 10^{-5} M fluorochrome solutions in 0.01 M phosphate buffer at pH 5.5. As an imitation to the labeling of antibodies by fluorescein conjugates, fluorescein was adsorbed to onion cuticle and buffered at pH 7.5. Rinsing and mounting was done either in buffer or, alternatively, in buffer containing 0.02–0.10 M of $Na_2S_2O_4$ (Merck). Coverslips were sealed with nail polish.
The microscope was Leitz Ortholux-II Fluorescence (Ploem)/Interference-contrast hybrid. A Nikon camera setup cast a real image onto a screen, from the center of which emerged a fibre optics light guide to a Hamamatsu R928 photo-multiplier, installed in a PAR Model 1140A Quantum photometer. A high pressure 200 W Hg bulb emitted the excitation beam, from which the Hg-line exciting the most intense fluorescence in each respective dye was selected by filters. In this microscope of incident illumination, the intensity Io of excitation increases as the square of the objective magnification. With our present set of filters and with $\times 100$ objective, Io at which nearly all our experiments were done was too intense for the needs of fluorescence microscopy. Switching to $\times 40$ objective lowered Io by $(40/100)^2 = 0.16$.
The figure shows representative fading curves, i.e., plots of fluorescence intensity vs time. With the exception of quina-

Fluorescence intensity (normalized) after 2 min of excitation by Hg line chosen for maximum fluorescence (conditions as in the figure)

Fluorochrome	F (2 min) / F(0) Buffer	$+Na_2S_2O_4$
Fluorescein[a]	0.67	1.00
Acridine orange	0.55	1.00
33258 Hoechst[b]	0.87	1.00
Acriflavine	0.22	0.90
Ethidium-Br[7]	0.27	0.87
Berberine	0.92	1.00
Quinacrine · 2 HCl	0.64	0.65
Rhodamine 3G[c]	0.54	0.62
ANS[11]	<0.1	<0.1
Quercetin-Al^{3+}[d]	(0.30)	(0.88)

[a] Adsorbed to onion skin and buffered at pH 7.5. [b] Courtesy of Dr H. Loewe, Farbwerke Hoechst, Frankfurt a. M. 80 (FRG). [c] Covalently bound to nuclei by Schiff-SO_2 reaction. [d] Excitation intensity $\times 0.16$ of that used for all other samples.