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Response to Letter of Dr. Mathur, *et al.*

To the Editor:

We are much pleased to learn that Dr. Mathur, *et al.* had different viewpoints and comments regarding our paper, "Preliminary Observation on Langerhans' Cells" ([1]). After our paper was published in September of 1982, we read some new articles relevant to that topic. Dr. Van Voorhis, *et al.* ([3]) studied 8 of 21 patients with leprosy (including 10 lepromatous cases, 5 borderline, and 6 tuberculoid) and found increased numbers of OKT6-positive cells in clusters in the epidermis just above the dermal infiltrates. On the other hand, the results in an earlier paper by Dr. Mathur, *et al.* ([2]) were contrary to our preliminary observation.

From the Letter to the Editor we are happy to learn that Dr. Mathur, *et al.* have done a lot of work on Langerhans' cells in leprosy, which will be very helpful to our further research. Recently we observed Langerhans' cells in seven cases of TT and BT using OKT6. The results were generally similar to that of our previous work, but some results were similar to that of Dr. Van Voorhis. The paper on this experiment will be submitted for publication to the Journal in the near future. Since the number of Langerhans' cells varies between 460–1000 per mm^2 of epidermis in man and there are regional variations in their distribution, the use of an autogeneous control with the epidermis from the same individual and from the same site must be an important factor in the correctness of experiments, and the results obtained using this method would be comparable. However, the common idea that Langerhans' cells are involved in the pathogenesis of leprosy is shared with Dr. Mathur and us.

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Fluorescence Microscopy of the Fluorescent Leprosy Antibody Absorption Test (FLA-ABS)

To the Editor:

The fluorescent leprosy antibody absorption test (FLA-ABS) for the detection of infection with *Mycobacterium leprae* by immunofluorescence microscopy has been originally described by Abe and coworkers ([1]). Several important factors that may influence the results and consequently the

utility of the test have already been reported ([2]). Despite this information and further details on the necessary equipment, kindly provided by Dr. Masahide Abe (National Institute for Leprosy Research, Tokyo, Japan) to one of us during a visit to his laboratory, we were unable to perform the test in our laboratory (Royal Tropical Institute, Amsterdam, The Netherlands) with satisfactory results. A major problem appeared to be the special fluorescence microscopy, using transmitted illumination, advised by Abe for the readout of the FLA-ABS test. Since, in our opinion, fluorescence microscopy with incident illumination would have advantages in terms of brightness and ease of operation, we tried to use incident illumination for the FLA-ABS test. However, we were not able to obtain the same results obtained by Abe using transmitted illumination. Since our problems were encountered by others as well (personal communication), we carefully investigated the fluorescence microscopy involved in the FLA-ABS test.

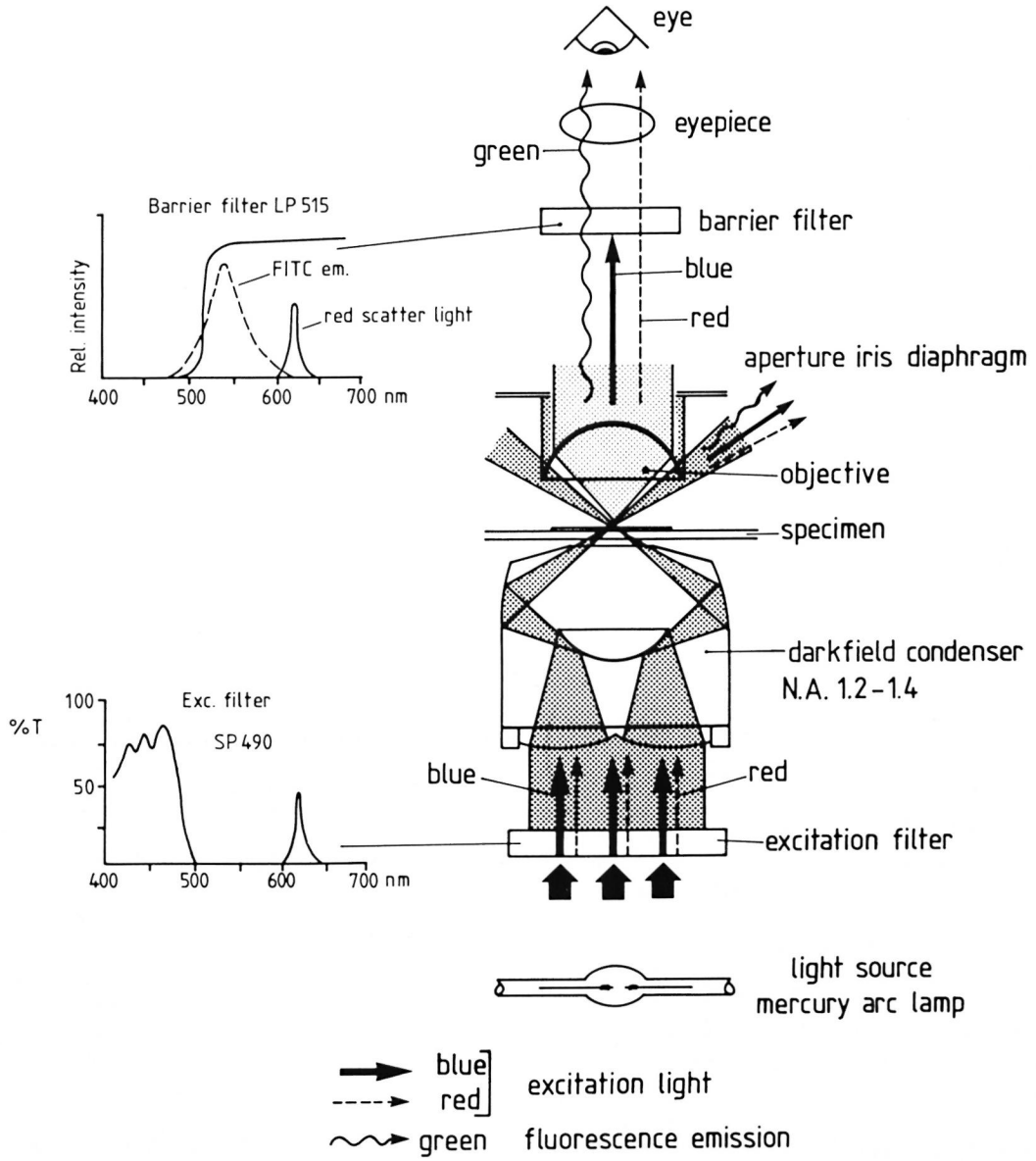
Abe and colleagues used a Tiyoda Model FM 200 fluorescence microscope, equipped for transmitted darkfield illumination for the readout of the FLA-ABS test ([1]). In the case of positive sera, strong green fluorescing bacteria were observed at blue excitation (BV filter). With the aid of an interference excitation filter AIF and FIF (Tiyoda Optical Co., Tokyo, Japan), the readout of the test may be simplified. In addition to the characteristic green FITC fluorescence of positive bacteria, a red autofluorescence was observed for negative bacteria, according to Abe, *et al.* ([1]).

However, when examining immunofluorescently stained *M. leprae* under a fluorescence microscope with epi-illumination, we did not observe the red autofluorescence of negative bacteria. The green FITC fluorescence of bacteria was, in the case of positive sera, very bright. No other filter systems tested (both glass filters and interference filters) for excitation wave lengths from UV to green resulted in red autofluorescence from bacteria. The specimens were then examined under transmitted illumination using a Tiyoda oil immersion darkfield condenser N.A. 1.23–1.46, with the same excitation and emission filters as used for incident illumination (obviously placed in the appropriate light pathways). A good FITC fluorescence was observed but no autofluorescence could be seen. Testing of other filters had no effect.

We then became suspicious about this "red autofluorescence" and started investigating the possibility of the scatter of red excitation light by the bacteria. A schematic representation of transmitted darkfield illumination is given in The Figure. In cases where no objects are present in the microscopic field, no light will enter the objective (so called darkfield). When microscopic objects are present, two types of radiation may enter the objective: fluorescence and scattered excitation light. In the case of FITC-stained objects, one deals with green fluorescence and, ideally, with only blue scattered excitation light. Of these two, only the green FITC fluorescence will pass the emission (barrier) filter. However, if excitation light consists also of some red light apart from the main component of blue light, red scattered light may be observed through the barrier filter. Some interference filters for excitation of FITC do transmit a considerable amount of red light in addition to blue light, strongly depending on their fabrication.

We therefore repeated the darkfield fluorescence microscopy (FM) with several interference filters for excitation of FITC from different manufacturers. The transmission characteristics of all filters were measured on a double-beam spectophotometer. Indeed, with the use of red-passing excitation filters, red bacteria could be observed. Optimal results were obtained with a Leitz 100× NPL fluotar objective N.A. 0.6–1.32 (variable aperture diaphragm) and a Tiyoda oil immersion darkfield condenser N.A. 1.23–1.46. In the case of FITC-positive bacteria, the red reflection was dominated to various degrees by the FITC fluorescence, as described by Abe, *et al.* ([1]).

We therefore concluded that the interference filter for FITC (Tiyoda) used by Abe and coworkers probably has a considerable transmittance in the red (between 600 nm and 650 nm). It is also possible that this filter has been developed on purpose for transmitted FM only, to visualize the non-fluorescing background in contrast to FITC-positive areas in thin sections. A comparable filter is being manufactured by



THE FIGURE. Schematic representation of fluorescence microscopy of the FLA-ABS test based on transmitted darkfield illumination. Blue (and red) excitation light enters the objective due to reflection on microscopic particles. The barrier filter blocks the blue excitation light but passes the red unwanted excitation light. Transmission characteristics of excitation and emission filters are shown at left in the figure.

Olympus Optical Co., Ltd., Tokyo, Japan ([3]).

The influence of this reflection in FM with incident illumination is different. The largest part of the red reflection will be filtered out by the chromatic beam splitter. Secondly, the 180° back-scatter intensity in incident illumination is several orders of magnitudes smaller than the near-forward angle scatter in FM with transmitted illumination ([6]). This explains why the "red autofluorescence" of negative bacteria was never observed under incident illumination.

Conclusions

1) Reading the FLA-ABS test in red and green (negative-positive), as described by Abe, is only possible under darkfield trans-

mitted illumination with an excitation filter that in addition to blue light also passes red light.

2) The red "autofluorescence" of negative bacteria appears to be the reflection of "unwanted" red excitation light.

3) Fluorescence microscopy with incident illumination cannot be used for the reading of the FLA-ABS test in red and green.

4) Obviously, reading of only FITC fluorescence is quite possible using incident illumination. Phase contrast microscopy or a counterstain for all bacteria might then be used to visualize the negative bacteria as well.

5) With this basic understanding of the differences between transmitted light and incident light in reading the FLA-ABS test, results in terms of specificity and sensitivity should be comparable in the two systems.

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Serological Abnormalities in Japanese Patients with Borderline Leprosy

To the Editor:

It is well known that circulating immune complexes (CIC) and some autoantibodies may be present in sera of patients with lepromatous leprosy ([8, 9, 10]). We have already reported that CIC and antinuclear antibodies (ANA) were present in sera of patients with tuberculoid leprosy as well as lepromatous leprosy ([1, 2]). In spite of the accumulating evidence concerning the appearance of CIC and ANA in lepromatous and tuberculoid leprosy, there are few reports with special reference to CIC and ANA in borderline leprosy.

We collected samples from 25 patients with borderline leprosy (BT, BB, BL) ([11]) in Nagashima Aiseien, Okayama, Japan. The patient ages ranged from 30–72 years, and the clinical stage was regressive ([6]). They were under treatment with antileprosy agents. Patients with other infectious diseases, cancer, and liver diseases were excluded from this study. The Bacteriologic Index (BI) was checked when the blood samples were collected. Twenty-five sera were obtained as controls from healthy individuals whose ages ranged from 25–79 years.

CIC was determined by C1q solid phase assay (SPA) ([4]); the detailed procedures were