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OKT3: A MONOCLONAL ANTI-HUMAN T LYMPHOCYTE ANTIBODY WITH POTENT MITOGENIC PROPERTIES

JEAN P. VAN WAUWE, JAN R. DE MEY, AND JAN G. GOOSSENS

From the Department of Immunology and Oncology, Janssen Pharmaceutica Research Laboratories, B-2340 Beerse, Belgium

OKT3, a monoclonal anti-human T cell antibody (IgG2), was found to induce DNA synthesis in human peripheral lymphocyte cultures. OKT3 induced maximal mitogenesis at a concentration of 10 to 20 ng/ml and was about 20-fold more potent than PHA as a mitogen. No high-dose inhibition of thymidine incorporation was noticed at concentrations up to 2.5 μg OKT3/ml. The monovalent Fab fragment of OKT3 was also mitogenic but about 100 times less potent than the parent IgG. OKT3 appeared to be a T lymphocyte mitogen as only sheep red blood cell rosetting lymphocytes were responsive. Quantitative studies on the binding of ^{125}I-labeled Fab fragment of OKT3 to human lymphocytes showed an average of 5.1×10^4 receptor sites/cell with an association of about 10^8 M^{-1} at 37°C, with no heterogeneity of the cell binding sites. These data suggest a strong interaction of the monoclonal OKT3 with a limited number of identical T cell membrane receptors. As this interaction can trigger mitogenesis, the cell membrane determinant recognized by OKT3 could be described as a "T cell stimulation receptor." The mitogenecity of the univalent Fab fragment indicated that the triggering of lymphocytes is not solely dependent on cross-linking of these receptors.

Human peripheral T lymphocytes can be stimulated to mitosis by a variety of chemical agents. Apart from lectins and specific antigens, miscellaneous components such as sodium periodate (1), phorbol myristate acetate (2), calcium ionophore A 23187 (3), and galactose oxidase (4) have been shown to be mitogenic. This group of blastogenic compounds also comprises a range of polyspecific antisera produced by heteroimmunization of human peripheral lymphocytes or thymus cells (5–8). The mechanism of lymphocyte stimulation by these mitogens is still largely unknown. However, T cell phytomitogens are considered to initiate the mitosis of lymphocytes by binding with specific sites on the lymphocyte membrane (9), but the nature of these T cell receptors remains ill-defined, since the isolation of lymphocyte membrane glycoproteins revealed multiple lectin-binding specificities (10). Also, the question of whether monovalent lectins are mitogenic remains unanswered because of the unavailability of such compounds. It appears unlikely that the use of polyspecific anti-lymphocyte antisera could solve these problems: these antisera are not very potent as mitogens, and they contain a heterologous population of antibodies recognizing multiple lymphocyte determinants. As the lymphocyte stimulation by mitogens is generally considered as a model for immune activation and growth regulation, some clarification of the above-mentioned items seems pertinent. We therefore considered the use of monoclonal anti-human T lymphocyte antibodies as mitogenic probes. Such antibodies are reactive with distinctive lymphocyte surface antigens and should be ideal molecules to solve the problem of the monovalent lymphocyte stimulation if Fab fragments could be isolated. Using the hybridoma technique of Kohler and Milstein, Kung *et al.* (11) generated several monoclonal antibodies (designated OKT) against cell membrane determinants of human peripheral T lymphocytes. Preliminary screening of these monospecific antibodies indicated that OKT3, an IgG2 immunoglobulin (11), was by far the most potent human lymphocyte mitogen.

This paper describes the mitogenic effect of OKT3 and its Fab fragment on human peripheral mononuclear cells. Binding experiments with ^{125}I-labeled Fab fragment of OKT3 indicated the presence of a restricted number of high affinity OKT3 receptors on the T lymphocyte, which could function as T cell stimulation sites.

MATERIALS AND METHODS

Materials. Lyophilized ascites fluids from mice injected either with p3 × 63 AgU_1 myeloma cells or with mouse spleen cell-myeloma hybrid cells producing the OKT3 antibody were made available by Dr. G. Goldstein and Dr. P. Kung (Ortho Pharmaceutical Co., Raritan, New Jersey). Concanavalin A (Con A) and phytohemagglutinin (PHA) were obtained from Miles-Yeda (Rehovot, Israel) and Burroughs-Wellcome (Erembodegem, Belgium), respectively. Protein A-Sepharose CL-4B was from Pharmacia (Gent, Belgium). ^{125}I-sodium iodide (100 mCi/ml) and ^{3}H-thymidine (21 Ci/mmole) were from Radiochemical Centre (Amersham, U. K.). Cold thymidine was obtained from Aldrich (Beerse, Belgium) and was used to prepare low specific activity ^{3}H-thymidine (2.25 Ci/mmole).

Purification of the IgG fraction from the ascites fluids. The IgG fraction of the ascites fluids (either OKT3 or control myeloma) was purified by affinity chromatography on a protein A-Sepharose column (12). Lyophilized ascites fluid was dissolved in distillated water at a concentration of 30 mg/ml and incubated for 15 min at room temperature with washed protein A-Sepharose (250 mg dry gel/ml of sample). This mixture was then poured into a column and eluted with 10 mM Tris-buffered saline, pH 7.6, until the extinction at 280 nm was lower than 0.02. The bound IgG was eluted with 0.1 M glycine-HCl, pH 2.8. Eluted peak fractions were pooled, neutralized, and dialyzed against Tris-buffered saline, pH 7.6. IgG concentrations were determined at 280 nm by using an extinction coefficient $E^{1\%}_{1\,cm}$ of 14.3 (13). About 10 to 12 mg purified IgG/ml ascite fluid was recovered.

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To prepare the Fab fragment, 30 mg of purified IgG was digested with 0.3 mg papain (Merck) for 4 hr at 37°C as described by Porter (14). After dialysis, the digest was applied to a protein A-Sepharose affinity column. The unbound fraction (Fab fragment) was rechromatographied on a new affinity column to eliminate contaminating traces of undigested IgG and Fc fragment. Fab concentrations were determined by absorption at 280 nm using an $E^{1\%}_{1\,cm}$ value of 15.3 (13). The isolated IgG fractions and the Fab preparation were sterilized by filtration (Millipore 0.22 μ, Amicon Co.), aliquoted, and stored at −18°C.

Polyacrylamide gel electrophoresis. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS)[1] was performed according to Laemmli (15).

Cell separation. Human peripheral blood mononuclear cells from adult, normal donors were obtained by dextran sedimentation and Ficoll-Hypaque gradient centrifugation (16). Cells recovered from the interface were washed twice with Hanks' balanced salt solution (HBSS) and suspended in RPMI 1640 medium supplemented with 100 units/ml penicillin and 100 μg/ml streptomycin together with 10% heat-inactivated FCS (Gibco, Gent, Belgium). Cell suspensions thus prepared were 80 to 85% lymphocytes and 15 to 20% monocytes by morphologic criteria.

Isolation of T cells, B cells, and monocytes. A T cell-enriched population was obtained after spontaneous rosette formulation: 5×10^6 mononuclear cells suspended in 1 ml were mixed with an equal volume of a suspension of 2×10^8 sheep red blood cells and incubated for 10 min at 37°C. After centrifugation at 200 × G for 5 min, the cell mixture was incubated for 1 hr at 4°C. The cells were then resuspended, layered on Ficoll-Hypaque, and centrifuged at 400 × G for 10 min at 4°C. The rosetted cells in the pellet were collected, the red cells were lysed in 0.87% Tris-buffered NH_4Cl, and the resulting T cell-enriched population was washed and suspended in culture medium containing 5% FCS. This cell preparation consisted of more than 95% rosetting cells as determined by rosetting and will be referred to as T cells.

Nonrosette-forming cells at the interface were rerosetted and separated on Ficoll-Hypaque gradient. Floating cells were depleted of monocytes by adherence to Petri dishes at 37°C for 1 hr in a humidified 5% CO_2-air. The nonadherent cells were collected and found to contain 60 to 70% surface immunoglobulin-positive cells by immunofluorescence by using fluorescein isothiocyanate- (FITC) conjugated polyvalent goat anti-human Ig antiserum (Nordic, Belgium), less than 2% rosette-forming cells, and not to be responsive to Con A or PHA. This cell population will be referred to as B cells. Adherent cells were removed by the lidocaine method as described (17) and contained more than 90% endogenous peroxidase-staining cells. These cells were used as a source of monocytes. In some experiments, the plastic adherence step was carried out before rosetting fractionation of the mononuclear cells. In this case, a comparable qualitative yield of lymphocyte subpopulation was obtained.

Cell cultures. Cultures were established in 16 x 25 mm plastic tubes (Falcon 3033). Lymphocytes were cultured in RPMI 1640 containing penicillin-streptomycin and 5% FCS at a concentration of 10^5 or 2×10^5 cells per tube in a volume of 200 μl. Appropriate concentrations of mitogens in 20 μl of medium were added at the initiation of the culturing period. When culturing purified T or B lymphocytes, cultures were always reconstituted with 2×10^4 monocytes per tube. Cells were incubated at 37°C in a 5% CO_2-atmosphere. Unless otherwise stated, cells were pulsed with 1 μCi of ^{3}H-thymidine (specific activity 2.25 Ci/mmole) in 20 μl of medium after 2 days of incubation. Twenty-four hours later the incorporation into DNA was assessed. Cells were washed once with 2 ml 0.9% NaCl and twice with 1 ml 5% trichloroacetic acid. The resulting precipitate was dissolved in 0.3 ml 0.5 N NaOH, transferred to counting vials, and 10 ml Instagel were added. Incorporation was measured by using a Packard Tri-Carb liquid scintillation spectrometer.

Iodination of OKT3 and its Fab fragment. OKT3 (100 μg) and the Fab fragment (150 μg) were iodinated with 500 μCi of ^{125}INa by using the chloramine-T method (18). The recovery of the iodinated proteins was determined as described (19) by adding 100 μl of a 2% bovine serum albumin (BSA) solution (protein concentration determined by the method of Lowry *et al.* (20) to the quenched reaction mixture. After Sephadex G-25 chromatography and dialysis, the total protein concentration was measured. Assuming that the concentration ratio of BSA to ^{125}I-protein had remained the same as in the reaction solution before gel filtration and dialysis, the amount of recovered iodinated protein could be calculated. The specific activity ranged from 2.10 to 2.35×10^6 cpm/μg for ^{125}I-OKT3 and from 1.54 to 1.70×10^6 cpm/μg for ^{125}I-Fab.

Binding studies. For quantitative binding experiments 200 μl of cell suspension (1.25×10^6 cells/ml) suspended in HBSS containing 0.2% BSA and 0.2% sodium azide were placed into 75 x 11 mm round-bottomed tubes containing 200 μl samples of known concentrations of ^{125}I-OKT3 or ^{125}I-Fab. After 30 min incubation of 37°C, cells were washed twice with 2 ml HBSS at room temperature. The cell pellet was then resuspended in 1 ml HBSS and radioactivity was counted by using a gamma spectrometer. The total amount of iodinated protein bound was corrected for nonspecific binding by substraction of the quantity of radioactivity bound in the presence of a 15-fold excess of cold OKT3. The association constant and number of binding sites per cell were calculated according to Scatchard (21).

RESULTS

Characterization of the purified OKT3 and its Fab fragment. Purified OKT3 and its Fab and Fc fragments were subjected to polyacrylamide gel electrophoresis in the presence of SDS. A typical electrophoresis pattern is seen in Figure 1. OKT3 showed one type of heavy chain (m.w. ~52,000) and one light chain (m.w. ~27,000). Neither the Fab nor the Fc piece revealed the presence of a band corresponding to the intact heavy chain, indicative for the complete digestion of the parent IgG. Moreover, double immunodiffusion analysis by using a Fab solution at 6 mg/ml and a rabbit anti-mouse IgG2 (Fc fragment) antiserum (Nordic, Belgium) showed no precipitin band.

Mitogenic effect of OKT3 on peripheral mononuclear cells. Figure 2 compares dose-response curves for the mitogenic effect of OKT3, PHA, Con A, and myeloma IgG on peripheral blood mononuclear cells. OKT3 showed already definite mitogenic activity at a concentration as low as 1 to 5 ng/ml (1 to 3×10^{-11} M). Maximal stimulation occurred at 10 to 20 ng/ml (0.7 to 1.2×10^{-10} M) for OKT3, 250 ng/ml (2.5×10^{-9} M) for PHA, and 5,000 ng/ml (4.1×10^{-8} M) for Con A. Myeloma IgG failed to stimulate these peripheral cells at 25 μg/ml. In contrast to the phytomitogens, OKT3 showed a broad maximum of stimulation displaying virtually no inhibition of the response at high concentrations up to 200 ng/ml. The same pattern of response,

[1] Abbreviations used in this paper: SDS, sodium dodecyl sulphate; HBSS, Hanks' balanced salt solution; FITC, fluorescein isothiocyanate.

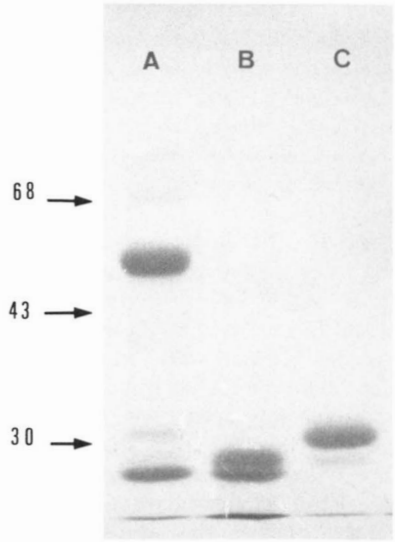

Figure 1. Polyacrylamide gel electrophoresis in the presence of SDS and 20 mM mercaptoethanol of 20 μg purified OKT3 (*A*), its Fab fragment (*B*), and its Fc fragment (*C*). Bands were resolved on a 10% acrylamide gel. Molecular weight standards used were: soybean trypsin inhibitor (21,000), carbonic anhydrase (30,000), ovalbumin (43,000), and bovine serum albumin (68,000).

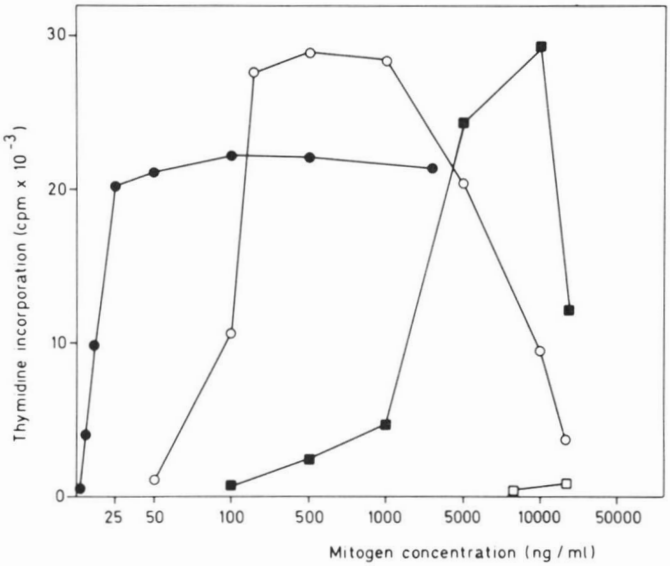

Figure 2. ^{3}H-thymidine incorporation into human peripheral blood mononuclear cells stimulated by varying concentrations of OKT3 (●), PHA (○), Con A (■), and myeloma IgG (□). Culture tubes contained 10^5 cells suspended in 0.2 ml of medium. Mitogens were added in 20 μl of medium. Cells were cultured for 3 days and pulsed with ^{3}H-thymidine (2.25 Ci/mmole) 24 hr before the termination of the culturing period. Each point represents the mean value of triplicate cultures.

characterized by the high potency of OKT3 and the absence of high-dose inhibition, was found when peripheral blood cells from 12 different donors were cultured in the presence of OKT3 (data not shown). The mitogenic activity expressed by the maximal amount of ^{3}H-thymidine was usually less pronounced for OKT3 than for PHA or Con A.

The kinetic of DNA synthesis by blood mononuclear cells stimulated by OKT3 is depicted in Figure 3. At both mitogen concentrations (12.5 and 5 ng/ml) the response was maximal after 72 hr of incubation.

Mitogenicity of the Fab fragment of OKT3. To determine whether OKT3 had to be divalent in order to exert mitogenic activity, we isolated its monovalent Fab fragment. Figure 4 shows the ^{3}H-thymidine incorporation when blood mononuclear cells were stimulated by different concentrations of OKT3 and its Fab fragment. As can be seen, the Fab fragment of OKT3 induced proliferation of mononuclear cells. However, its pattern of mitogenicity was quite different from that of the parent IgG: the Fab fragment was about one-third less active than OKT3 (maximal ^{3}H-thymidine incorporation 31,300 ± 1,815 cpm for the OKT3 *vs* 10,580 ± 518 cpm for the Fab fragment). When the minimal effective mitogenic doses were compared, the Fab fragment (100 ng/ml) was approximately 100 times less potent than the undigested IgG (1 ng/ml).

Mitogenic effect of OKT3 on purified T and B cells. In order to characterize the responsive cell type in the peripheral blood,

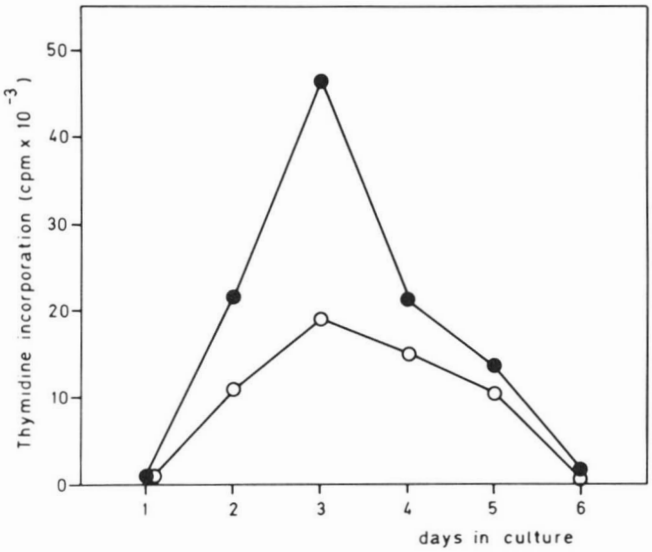

Figure 3. Kinetic of the ^{3}H-thymidine incorporation into peripheral blood mononuclear cells by OKT3 at 12.5 ng/ml (●) and 5 ng/ml (○). Culture tubes contained 10^5 cells and were incubated from 1 to 6 days. ^{3}H-thymidine (21 Ci/mmole) was added 4 hr before cell harvest at the time points indicated. Each point represents the mean value of triplicate cultures.

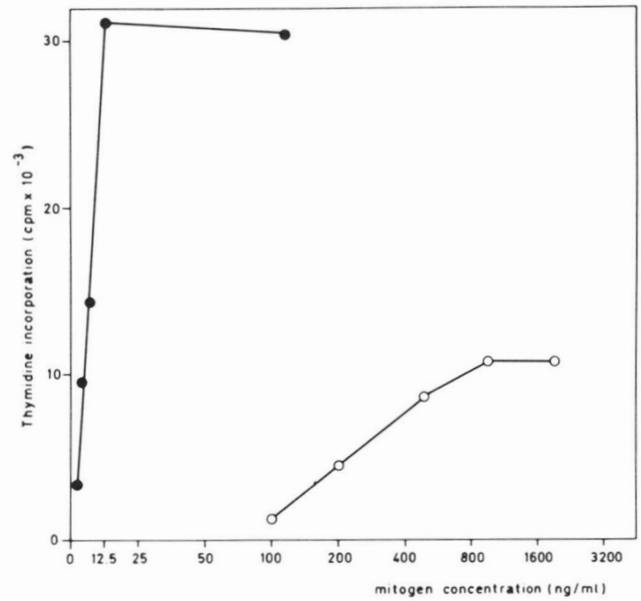

Figure 4. ^{3}H-thymidine incorporation into human blood mononuclear cells by varying concentrations of OKT3 (●) and its Fab fragment (○). Same experimental procedures as in Figure 2.

the stimulatory effect of OKT3 on unfractionated purified T and B cells were examined (Table I). OKT3 induced substantial ^{3}H-thymidine incorporation into unfractionated and purified T cells but not into the B cell subpopulation. As such, OKT3 had an identical pattern of activity to PHA and could be identified as a T cell mitogen.

Binding of OKT3 and its Fab fragment to purified T lymphocytes. The high potency together with the absence of high-dose inhibition of the OKT3 mitogenicity on peripheral T lymphocytes suggested a strong specific T cell binding together with a restricted number of cell surface receptor sites for this antibody. Incubation of ^{125}I-OKT3 (100 and 200 ng) with either B cells or monocytes failed to show an appreciable amount of bound radioactivity to these cells (Fig. 5). However, incubation of increasing concentrations of ^{125}I-OKT3 with purified T lymphocytes showed saturation binding. This interaction was highly specific, inasmuch as the binding of ^{125}I-OKT3 could be completely inhibited by the addition of a 15-fold excess of unlabeled OKT3. In order to quantitate the number of OKT3 determinants on the T cell surface and to estimate the association constant, binding experiments were performed by using the ^{125}I-Fab fragment of OKT3 as a monovalent ligand. Applying the Scatchard relation $r/c = nK - rK$, where r is the amount of ^{125}I-Fab fragment bound, c is the free ^{125}I-Fab fragment concentration, K, the association constant, and n, the number of binding sites per cell could be calculated. If the lymphocyte binding sites are homogenous, a plot of r/c against r yields a straight line with the ordinate intercept at $r = n$ and a slope equal to $-K$. As shown in Figure 6, our binding results do indeed give such straight line, from which it was calculated that about 5 ng/ml of Fab fragment was bound to 1.25×10^6 lymphocytes with an association constant, K, of 10^8 M^{-1}. Assuming a m.w. of 50,000 for the Fab fragment of OKT3, the average of 5.1×10^4 binding sites per T lymphocyte was found.

DISCUSSION

The present experiments demonstrate that OKT3, a monoclonal anti-human T cell antibody, is a potent mitogen for human peripheral mononuclear cells. OKT3 showed maximal stimulation from concentrations as low as 10 to 20 ng/ml on and was about 10 and 100 times more potent than PHA and Con A, respectively. Unlike PHA and Con A, OKT3 was active over a large concentration range: no pronounced decrease of the mitogenic response was observed from 50 ng/ml to over 2000 ng/ml. Either the divalent nature and/or the monoclonality of OKT3 could explain this absence of high-dose inhibition.

TABLE I

Mitogenic effect of CKT3 and PHA on unfractionated, purified T and B lymphocytes[a]

Mitogen (Concentration)	^{3}H-Thymidine Incorporation		
	Unfractionated lymphocytes	T lymphocytes	B lymphocytes
	1.783 ± 20	450 ± 56	1,785 ± 104
OKT3 (100 ng/ml)	38,071 ± 1,572	41,547 ± 872	5,454 ± 405
PHA (500 ng/ml)	57,600 ± 804	62,340 ± 918	3,415 ± 210

[a] Ficoll-Hypaque-isolated mononuclear cells were monocyte depleted before rosetting with sheep red blood cells. Lymphocytes were cultured at 2×10^5 cells/tube after readdition of monocytes (2×10^4 cells/tube) in the presence of medium or mitogen. ^{3}H-thymidine (2.25 Ci/mmole) was added 24 hr before all harvesting. Figures are the mean values ± S.E.M. for triplicate cultures.

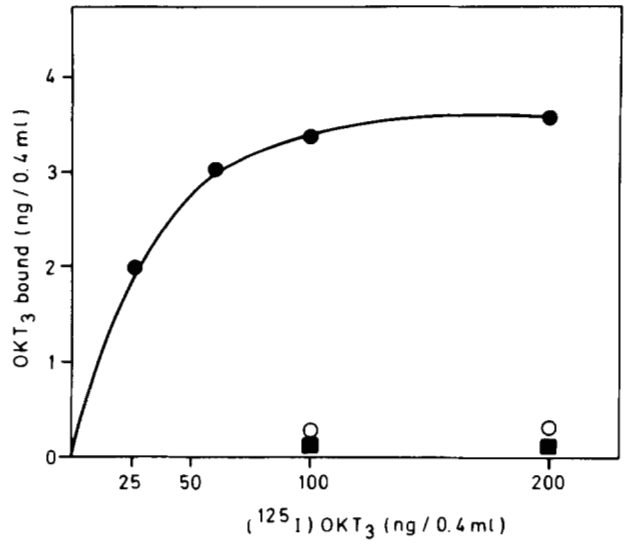

Figure 5. Specific binding of ^{125}I-OKT3 to purified T and B lymphocytes and monocytes. Varying quantities of ^{125}I-OKT3 (specific activity of $2.25 \times 10^6/\mu g$) were incubated for 30 min at 37°C with 5×10^5 T cells (●), B cells (○), or monocytes (■). Total incubation volume was 0.4 ml HBSS containing 0.2% BSA and sodium azide. Points are means of duplicate values.

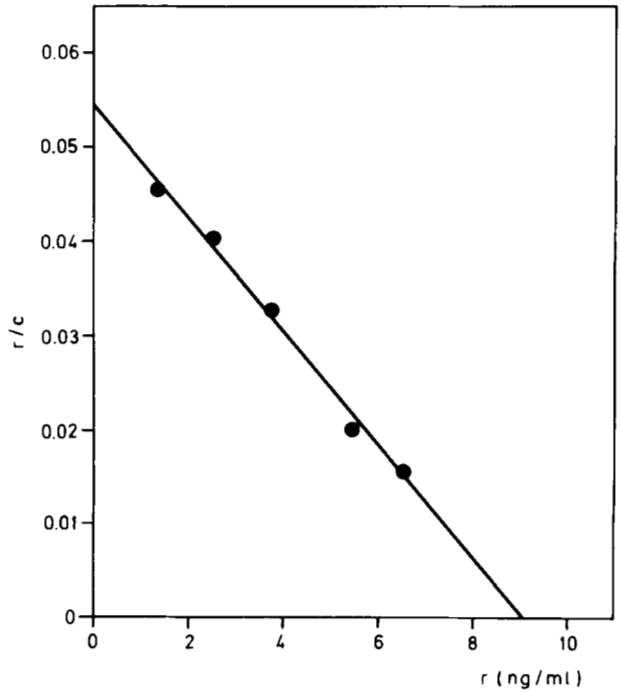

Figure 6. Scatchard plot of the binding of ^{125}I-Fab fragment to purified T lymphocytes. Varying quantities of ^{125}I-Fab, ranging from 30 to 480 ng, were incubated with 5×10^5 purified T cells for 30 min at 37°C. Total incubation volume was 0.4 ml HBSS containing 0.2% BSA and sodium azide. Points represent the mean of duplicate determinations from two independent experiments.

In fact, identical wide dose-response curves have been reported for other divalent mitogens, such as succinyl Con A (22) and the IgG fraction of carbohydrate specific antiserum (23). Moreover, due to its monospecificity, OKT3 recognized only one distinct cell surface determinant on the lymphocyte membrane. When these cell receptors are occupied, no further binding between OKT3 and the lymphocyte occurs, which could give rise to inhibitory effects of the cellular response.

Papain digestion of OKT3 yielded a monovalent Fab fragment that was still capable of inducing ^{3}H-thymidine incorporation into peripheral lymphocytes. The possibility that the stimulatory effects of the Fab preparation was caused by a minor contamination with undigested IgG was excluded: SDS polyacrylamide gel electrophoresis together with double immunodiffusion analysis of the Fab fragment did not reveal the presence of intact heavy chain.

Also, the characteristics of the dose-response curves of OKT3 and its Fab fragment were clearly discernable: both the mitogenic activity and potency of the Fab fragment were less pronounced than in the case of OKT3. For instance, when compared on a molar basis, the stimulatory efficacy of the Fab fragment was 300 times less than the parent IgG. Obviously, the intrinsic capacity of divalent OKT3 to cross-link and aggregate lymphocyte receptors greatly contributes to its mitogenic potency. However, the finding that the monovalent Fab fragment of OKT3 was stimulatory indicates that cross-linkage of cell receptors is not a *conditio sine qua non* for triggering lymphocyte proliferation. This conclusion is in disagreement with earlier findings that suggested a divalency requirement for anti-lymphocyte antibodies in order to be mitogenic (8, 24, 25). The reason for this discrepancy could be owing to the weak mitogenic potency of the antibodies and to the relatively low concentrations of their monovalent fragments used in the latter studies. For instance, the mitogenicity of the Fab' fragment of the anti-lymphocyte antibody described by Woodruff *et al.* (8) was tested at a highest concentration of 2.5 mg/2.5 ml of culture, which was only 20 times larger than the minimal effective dose of the parent IgG (0.1 mg/2.5 ml culture). On the other hand, our finding was not unique, since the Fab preparation of anti-rabbit immunoglobulin allotype antibodies was reported to be mitogenic for rabbit lymphocytes (26). Apparently, some, but not all, lymphocyte membrane sites can interact with monovalent mitogenic molecules resulting in triggering the mitotic sequence of the lymphocyte. Thus, the OKT3 receptor appears to belong to this particular class of lymphocyte determinants.

In order to delineate the cell responsive to OKT3, T and B lymphocytes were isolated by rosetting with sheep red blood cells. OKT3 only stimulated purified T lymphocytes and could therefore be classified as a T cell mitogen. This conclusion was in keeping with the known T cell specificity of this antibody (11) and with our findings that ^{125}I-OKT3 was only reactive with T cells, and not with B cells or monocytes.

Our data on the binding of ^{125}I-labeled Fab fragment of OKT3 to T lymphocytes indicated extremely strong binding with an association constant of 10^8 M^{-1}. This value is about 2 to 10 times greater than for the binding of phytomitogens to human T lymphocytes: PHA and Con A had K values of 5.1×10^7 and 8.8×10^6 M^{-1}, respectively (27). OKT3 interacted with a restricted number of T lymphocyte membrane receptors, averaging 5.1×10^4 per cell. This number is comparable to the average density of Fc receptors for cross-linked trimers of IgG on human peripheral blood lymphocytes and monocytes (28), but is remarkably limited when compared with the number of phytomitogen binding sites per T lymphocyte (27). PHA ($n = 3.3 \times 10^6$), lentin lectin ($n = 2.9 \times 10^6$), and Con A ($n = 1.7 \times 10^6$) have about 50 times more interaction sites per cell than OKT3. Moreover, from the linearity of the Scatchard plot and the monoclonal nature of OKT3, the chemical uniformity of the OKT3 receptors could be postulated.

Considering both the high mitogenic potency of OKT3 and its binding to a unique T cell surface determinant, it seems plausible to describe the OKT3 lymphocyte receptor as a "T cell stimulation" receptor.

Apart from its clinical merit for studying the quantity and function of T lymphocytes, OKT3 could also be of experimental value. For instance, the isolation and characterization of the OKT3 receptor site should yield important information on the mechanism of lymphocyte activation. Also, due to its high T cell-binding specificity, this mitogen could be invaluable for the elucidation of T-B lymphocyte cooperative phenomena.

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