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"Infectious" Transplantation Tolerance

Shixin Qin, Stephen P. Cobbold, Heather Pope, James Elliott, Dimitris Kioussis, Joanna Davies, Herman Waldmann*

The maintenance of transplantation tolerance induced in adult mice after short-term treatment with nonlytic monoclonal antibodies to CD4 and CD8 was investigated. $CD4^+$ T cells from tolerant mice disabled naïve lymphocytes so that they too could not reject the graft. The naïve lymphocytes that had been so disabled also became tolerant and, in turn, developed the capacity to specifically disable other naïve lymphocytes. This process of "infectious" tolerance explains why no further immunosuppression was needed to maintain long-term transplantation tolerance.

A major goal of transplantation is that the recipient should accept and become tolerant to a foreign organ graft as though it were a "self" tissue. The classic experiments of Medawar and colleagues (*1*) established this principle in the neonatal mouse. In the adult mouse lifelong tolerance can also be achieved with short courses of monoclonal antibodies (MAbs) to CD4 plus CD8 (*2–4*) or CD11a plus intercellular adhesion molecule-1 (ICAM-1) (*5*), even though new T cells continue to be made by the thymus. We wished to determine the mechanisms that might operate to keep the proliferation of such new T cells in check. A clue was provided by the finding that we could not break tolerance by transfusions of normal naïve lymphocytes (*3*). Consequently, we reasoned that if we could establish the mechanism by which these naïve lymphocytes become disabled, we might understand the processes controlling any new T cells produced by the body.

In order to follow events in a stable peripheral T cell pool, we used mice that had been thymectomized (ATx) at 4 to 6 weeks of age. Such ATx CBA/Ca mice

S. Qin, S. P. Cobbold, H. Pope, J. Davies, H. Waldmann, Department of Pathology, University of Cambridge, Cambridge CB2 1QP, United Kingdom.
J. Elliott and D. Kioussis, Laboratory of Molecular Immunology, National Institute for Medical Research, Mill Hill, London NW7 1AA, United Kingdom.

*To whom correspondence should be addressed.

were transplanted with skin from minor histocompatibility–mismatched B10.BR donors and then given the tolerizing protocol (6). At 4 months after transplantation an infusion of 50 million splenocytes from normal CBA/Ca mice, together with fresh B10.BR grafts, could not break tolerance in any of the mice (Fig. 1A). However, prior ablation of the T cells of the tolerant host with depleting MAbs to CD4 and CD8 (7) 7 weeks before transplantation of the second graft resulted in the rejection of both the first and second grafts by the infused cells. This demonstrated that it was the T cells in the tolerant host that were inhibiting the infused cells.

Consistent with this finding, spleen cells from tolerant animals could directly suppress naïve lymphocytes in a classic adoptive transfer–type system. Fifty million spleen cells from each of tolerant and naïve animals were transferred into T cell–depleted "test-tube" mice (8) grafted with a test B10.BR skin and a third-party skin (Fig. 1B). Cells from tolerant donors prevented naïve cells from rejecting B10.BR grafts (9) but not the third-party grafts (10). Prior removal of $CD4^+$ but not $CD8^+$ T cells eliminated the suppression, so we concluded that $CD4^+$ T cells from the tolerant mice were necessary for the prevention of graft rejection.

In order to determine the fate of naïve T cells that had been unable to reject a graft in tolerant mice, we used transgenic mice ($hCD2^+$/CBA) as the tolerant hosts that express human CD2 on all of their T cells (11). This allowed us to identify the host T cells and to ablate them with MAbs to human CD2 as desired. These mice rejected B10.BR skin grafts normally with a median survival time (MST) of 14 days (Fig. 2A), could be made tolerant as before, and were once again nonpermissive to lymphocyte infusions from naïve nontransgenic littermates (Fig. 2B). If, at the time of infusion, the host-type $hCD2^+$ T cells were depleted with a MAb to human CD2 (12, 13), the infused T cells could reject both B10.BR and third-party BALB/c skin grafts promptly (MST = 14 and 13 days, respectively; Fig. 2B). This confirmed that T cells in the tolerant $hCD2^+$ recipient were disabling graft rejection by naïve cells and that infused donor T cells could otherwise function normally in the $hCD2^+$ recipient.

If we allowed naïve donor cells to coexist with tolerant host cells for 7 days and then depleted host T cells (with MAb to hCD2), the infused donor cells still rejected the B10.BR grafts in six of eight mice (Table 1, group C). However, when we extended the period of coexistence to 2 weeks, all mice kept their B10.BR grafts indefinitely yet rejected third-party grafts normally (Table 1, group B) (14). The

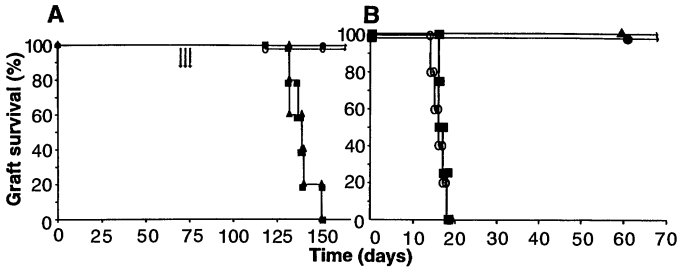

Fig. 1. $CD4^+$ cells in tolerant mice prevent naïve T cells from breaking tolerance. (**A**) ATx CBA/Ca mice were made tolerant on day 0 to B10.BR tail skin (6). On day 70, half of these tolerant mice were given three doses (over 2 weeks) of MAbs (↓) to deplete $CD4^+$ and $CD8^+$ cells (7). On day 119, all tolerant mice received 5×10^7 spleen cells intravenously from normal CBA/Ca mice. Another B10.BR and a third-party BALB/c skin graft were transplanted at this time. All BALB/c grafts were rejected within 14 days. Both original (●) and second (○) B10.BR skin grafts survived in mice whose T cells had not been depleted (n = 6), whereas mice that had been depleted of T cells (n = 5) rejected both the original (▲) and second B10.BR grafts (■). (**B**) Cells from the spleen and lymph node were taken from ATx CBA/Ca mice made tolerant to B10.BR skin (6) more than 90 days previously. $CD4^-$ and $CD8^-$ cells from tolerant mice were obtained as follows: ATx CBA/Ca mice made tolerant as above received two depleting doses of either CD4 or CD8 MAbs (7). Eight weeks later, cells from the spleen and lymph node of these mice were further depleted with biotinylated MAbs YTA 3.1 or YTS 156 plus streptavidin Dynabeads (Dynal, Oslo, Norway) such that each treated population contained <0.5% of $CD4^+$ or $CD8^+$ cells, respectively. Fifty million tolerant (▲), $CD4^-$ (○), $CD8^-$ (●), or no (■) cells were mixed with 5×10^7 normal spleen cells and injected into ATx T cell–depleted recipient CBA/Ca mice (8) that had been grafted with B10.BR and BALB/c skin the same day. The survival of B10.BR grafts is shown, and all third-party BALB/c grafts were rejected within 18 days (MST = 15 days). Separate experiments established that tolerant spleen cells alone also suppressed.

failure of infused cells to reject B10.BR grafts was not due to any lack of engraftment because 2 weeks after infusion of 50 million unmarked ($hCD2^-$) naïve splenocytes into tolerant $hCD2^+$/CBA recipients, donor T cells represented approximately 26% of the $CD3^+$ T cells in the peripheral blood (Fig. 3C). After treatment with the MAb to hCD2, all the peripheral T cells (>99%) were of donor ($hCD2^-$) phenotype (Fig. 3D). Therefore, during a 2-week period of coexistence with tolerant cells, the naïve donor T cells had themselves become tolerant.

We investigated this second-generation tolerance further. Tolerant mice that had been reconstituted with unmarked donor ($hCD2^-$) T cells (after depletion of $hCD2^+$ cells at 2 weeks) maintained their tolerant state even after a further infusion of 50 million naïve donor spleen cells (60 days after the first infusion). None of these animals rejected their two established B10.BR grafts or their fresh B10.BR graft (all six animals kept the three B10.BR grafts for >70 days after the second donor infusion). This result indicated that the acquisition of tolerance in naïve cells was again accompanied by the development of a capacity to prevent newly infused cells from rejecting the graft.

We have previously observed this same failure to break tolerance by lymphocyte infusions in antibody-facilitated tolerance to proteins and skin- or bone-marrow grafts (3, 15). It is also difficult to break (by lymphocyte infusion) neonatal tolerance (16) or peripheral tolerance in mice ex-

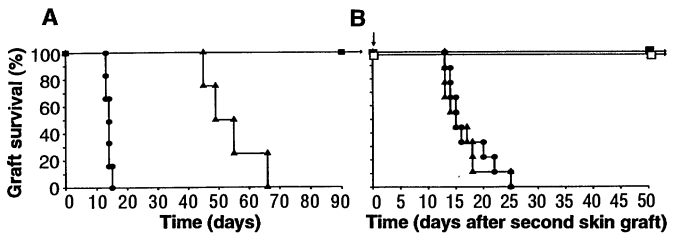

Fig. 2. Induction of tolerance and failure to break tolerance in $hCD2^+$/CBA mice. (**A**) Adult thymectomized $hCD2^+$/CBA mice rejected B10.BR tail-skin grafts (MST = 14 days) (●; n = 6). Intravenous injection of ATx $hCD2^+$/CBA mice at the time of skin grafting with YTH 655 MAb (1 mg per mouse) to deplete $hCD2^+$ cells (13) prolonged the MST of B10.BR skin grafts to >90 days (■; n = 5) and of BALB/c skin to 52 days (▲; n = 4). (**B**) ATx $hCD2^+$/CBA mice were made tolerant to B10.BR skin (6) at 10 to 12 weeks of age. A second B10.BR skin was grafted 70 to 90 days later (time indicated by arrow) to confirm tolerance (defined as day 0). Data were pooled from two experiments. Mice with no other treatment kept both original and test grafts beyond day 60 (■; n = 8). Injection at the time of the second skin grafting, with 5×10^7 normal spleen cells, from $hCD2^-$ littermates did not affect graft survival (□; n = 7). Another nine tolerant mice were depleted of host ($hCD2^+$) T cells with YTH 655 MAb at the time of donor cell infusion. Rejection of original (●; MST = 15 days) and second (▲; MST = 15 days) B10.BR skin grafts was only observed in these hCD2-depleted mice. Third-party BALB/c skin grafts given at the time of the second B10.BR graft were rejected in all groups within 15 days.

Table 1. Induction of tolerance in naïve spleen cells after coexistence with tolerant T cells. Adult thymectomized hCD2+/CBA transgenic mice (in groups B and C) were made tolerant to B10.BR skin by nondepleting antibody therapy (*6*). Mice in group A were untreated ATx hCD2+/CBA controls. All mice were given 5 × 10^7 spleen cells from hCD2− littermates at 56 or 63 days after tolerance induction. After a further period of 1 week (group C) or 2 weeks (groups A and B), the host hCD2+ cells were selectively depleted by two intravenous injections (1 mg each per mouse, 7 days apart) of YTH 655 MAb to hCD2 (*12, 13*). On the day of the second MAb injection, all mice were grafted with a fresh B10.BR (test) skin and third-party BALB/c skin. Graft survival is given for individual mice pooled from two separate experiments.

Group	Period of coexistence	Graft survival from time of test skin transplant (days)		
		Tolerizing B10.BR skin	Test B10.BR skin	Third-party BALB/c skin
A—control mice (not tolerant)	2 weeks (days −14 to 0)	Not grafted	13, 14, 14, 16	12, 13, 13, 13
B—tolerant mice	2 weeks (days −14 to 0)	All >60 (n = 8)	All >60 (n = 8)	13, 14, 14, 15, 16, 17, 17, 18
C—tolerant mice	1 week (days −7 to 0)	17, 18, 18, 19, 25, 25, >60, >60	17, 19, 18, 20, 24, 25, >60, >60	12, 12, 12, 12, 13, 14, 18, 19

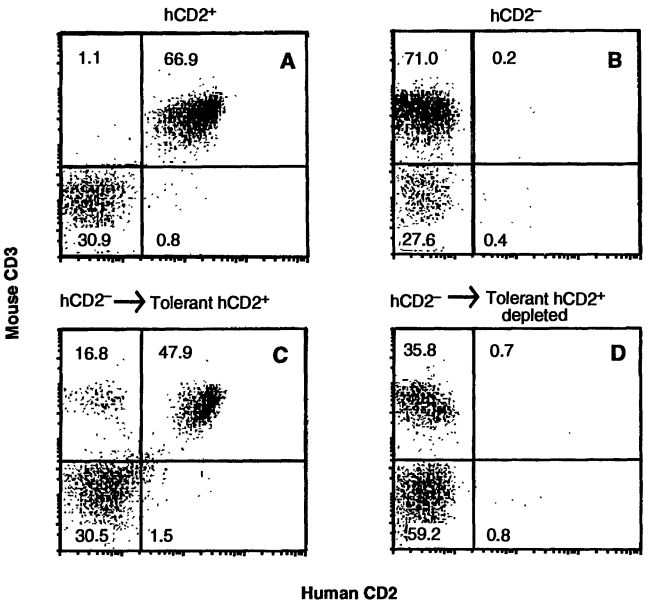

Fig. 3. Engraftment of infused hCD2− T cells in hCD2+/CBA mice. Two-color immunofluorescence of PBL (*18*) stained with rat MAbs to mouse CD3 and human CD2 from (**A**) untreated ATx hCD2+/CBA control mice; (**B**) untreated hCD2− littermate controls; (**C**) B10.BR-tolerant ATx hCD2+/CBA mice given 5 × 10^7 hCD2− spleen cells; and (**D**) B10.BR-tolerant ATx hCD2+/CBA mice given 5 × 10^7 hCD2− spleen cells and MAb to human CD2. Mean percentage staining is shown of three or four mice per group in each quadrant of the representative plots. The mean percentage (±SD) and geometric mean fluorescence (1 to 10,000 channels) of CD3+hCD2+ cells in (A) and (C), respectively, were 66.9 ± 4.6%, 415 (×/÷1.1), and 47.9 ± 8.0%, 398 (×/÷1.2). Mice in (D) were followed for 3 months after antibody treatment; however, we detected no CD3+CD2+ cells in the PBL (<1%) in any of these mice.

pressing transgene products extrathymically (*17*). By showing engraftment of the infused T cells, we have ruled out space constraints as an explanation and conclude that peripheral T cells in the tolerant host can guide new T cells infused from the outside toward a similar state of tolerance. Our findings may explain why euthymic mice exhibit long-term tolerance after short courses of nonlytic antibody therapy. A tolerant peripheral immune system would presumably deal with new T cells released from the thymus in the same ways as it deals with T cells introduced experimentally from the outside—that is, by "infectious" tolerance.

In addition to documenting transferable transplant suppression by CD4+ T cells, these findings show that the suppressed T cells are themselves guided to tolerance and ultimately can disable other T cells. Because second-generation tolerance arises in the absence of any MAb to CD4 or CD8, it probably represents a "natural" response of the immune system, which, once initiated, becomes self-sustaining.

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6. CBA/Ca, B10.BR, and BALB/c mice (Harlan Olac, Bicester, United Kingdom) were treated in accordance with the Home Office Animals (Scientific Procedures) Act of 1986. Adult thymectomy at 5 to 6 weeks of age and skin grafting at least 4 weeks later (*1, 3*) were done with Hypnodil/Sublimaze (Janssen) as anesthetic. For induction of tolerance to B10.BR skin, a mixture of 0.5 mg each of YTS 177.9 [nondepleting rat immunoglobulin G2a (IgG2a) MAb to mouse CD4 (*3*)] and YTS 105.18 [rat IgG2a MAb to mouse CD8 (*3*)] was injected into the tail vein of each mouse on days 0, 2, and 4 after skin grafting.
7. Each dose consisted of 1 mg of a cocktail of depleting rat IgG2b MAbs to CD4 (YTS 191 and YTS 3.1), CD8 (YTS 169 and YTS 156), or both injected into each mouse (*2*).
8. ATx CBA/Ca mice that had been depleted of T cells by treatment with MAbs to CD4 and CD8 (*7*) consistently did not reject B10.BR skin grafts (95% graft survival for >72 days over four experiments), and only a proportion of these mice rejected BALB/c skin grafts slowly (MST = 21 days with 45% survival for >36 days).
9. Tolerant cells could be given at 3 (but not 7) days after naïve cells and still suppress graft rejection indefinitely.
10. The ability of tolerant spleen cells to suppress naïve T cells on adoptive transfer into T cell–depleted ATx recipients has been independently confirmed by three workers in our laboratory with two different combinations of strains (B10.BR or BALB/k skin onto CBA/Ca recipient mice). The maintenance of tolerance after adoptive transfer requires antigen (that is, a skin graft) because if the test grafting is delayed beyond 4 months the skin is rejected (unpublished results).
11. hCD2+/CBA transgenic mice [G. Lang *et al.*, *EMBO J.* **7**, 1675 (1988)] have five to seven copies of the hCD2 transgene and were bred as hemizygotes in conventional animal facilities. Each mouse was screened for the expression of hCD2 on peripheral blood lymphocytes (PBL) by immunofluorescence staining with YTH 655 (*13*) and fluorescein isothiocyanate (FITC)–conjugated MAb NORIG 7.16 to rat IgG2b (SeraLab, Crawley Down, United Kingdom). The positive and negative offspring were used separately in sex- and age-matched groups in the experiments.
12. Host T cells were ablated by the MAb to hCD2 because normal hCD2+/CBA mice given the same MAb treatment all held B10.BR skin grafts for at least 90 days and BALB/c skin with MST = 52 days (Fig. 2A).
13. The rat MAbs to hCD2 were YTH 655 and YTH 616 (both IgG2b) [described in H. P. Tighe, thesis, Cambridge University (1987); E. P. Rieber, in *Leucocyte Typing*, W. Knapp *et al.*, Eds. (Oxford Univ. Press, London, 1989), vol. 4, pp. 229–249].
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18. PBLs were obtained by bleeding from the tail vein of untreated mice (Fig. 3, A and B) or 2 weeks after infusion of 5 × 10^7 splenocytes from hCD2− littermates (Fig. 3, C and D) and treatment with two intravenous doses (1 mg per mouse) of rat IgG2b MAb YTH 655 to human CD2, 1 week apart (Fig. 3D). The PBLs were separated on Ficoll-

Hypaque, stained with biotinylated MAb YTH 616 to human CD2 (*13*) and rat IgG2a MAb KT3 to mouse CD3 [K. Tomonari, *Immunogenetics* **28**, 455 (1988)], and detected by streptavidin R-phycoerythrin (SeraLab) and FITC-conjugated MAb MARG2A to rat IgG2a (Serotec, Oxford, United Kingdom), respectively. Labeled cells were analyzed on a fluorescence-activated cell sorter (FACScan; Becton Dickinson, Oxford, United Kingdom).

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Carboxyl Methylation of Ras-Related Proteins During Signal Transduction in Neutrophils

Mark R. Philips,* Michael H. Pillinger, Roland Staud,† Craig Volker, Melvin G. Rosenfeld, Gerald Weissmann, Jeffry B. Stock

In human neutrophils, as in other cell types, Ras-related guanosine triphosphate–binding proteins are directed toward their regulatory targets in membranes by a series of posttranslational modifications that include methyl esterification of a carboxyl-terminal prenylcysteine residue. In intact cells and in a reconstituted in vitro system, the amount of carboxyl methylation of Ras-related proteins increased in response to the chemoattractant *N*-formylmethionyl-leucyl-phenylalanine (FMLP). Activation of Ras-related proteins by guanosine-5′-*O*-(3-thiotriphosphate) had a similar effect and induced translocation of $p22^{rac2}$ from cytosol to plasma membrane. Inhibitors of prenylcysteine carboxyl methylation effectively blocked neutrophil responses to FMLP. These findings suggest a direct link between receptor-mediated signal transduction and the carboxyl methylation of Ras-related proteins.

The Ras-related guanosine triphosphate (GTP)–binding proteins regulate a wide variety of cellular processes, including signal transduction (*1*). The biological activity of Ras-related proteins depends on the ability of these proteins to associate with membranes. These intrinsically hydrophilic proteins become associated with membranes as a result of a series of posttranslational modifications of their COOH-termini (*2*). The Ras-related proteins of the Ras and Rho subfamilies are among a class of proteins that end with the COOH-terminal consensus sequence CAAX, in which C is cysteine, A is usually an aliphatic amino acid, and X is another amino acid. Several cytosolic prenyl transferases recognize these sequences and catalyze the attachment of a polyisoprene chain (15-carbon farnesyl or 20-carbon geranylgeranyl) by a thioether linkage to the cysteine. The prenylated COOH-terminus is recognized by a membrane-associated protease that removes the AAX amino acids. The new COOH-terminal prenylcysteine subsequently becomes a substrate for a membrane-associated carboxyl methyltransferase that methyl esterifies the α carboxyl group. Thus, prenylation and AAX proteolysis are prerequisites for carboxyl methylation.

Unlike prenylation and proteolysis, carboxyl methylation is reversible under physiologic conditions (*3*). Genetic studies in yeast (*4*) and in vitro analysis of $p21^{K\text{-}ras(B)}$ (*5*) indicate that carboxyl methylation augments the membrane association of processed CAAX-containing proteins, presumably by neutralizing the negative charge at the COOH-terminus. It is unclear whether the COOH-terminal prenylcysteine methyl ester inserts nonspecifically into lipid bilayers or binds to specific membrane targets. The reversibility of carboxyl methylation and the function of Ras-related proteins in signaling suggest that carboxyl methylation may regulate signal transduction.

Ras-mediated signaling has been studied in tissue culture, generally in the context of growth factor–mediated proliferation and differentiation. Unlike tissue culture cells, neutrophils are terminally differentiated, short-lived cells that have no proliferative capacity. Neutrophils have relatively well characterized pathways of signal transduction in which stimulus-response coupling is accomplished within minutes and does not require protein synthesis (*6*). We therefore used human neutrophils as a system in which to test the hypothesis that carboxyl methylation of Ras-related proteins is involved in signal transduction.

We first determined whether circulating neutrophils retain the capacity to process Ras-related proteins. Metabolic labeling of human neutrophils with [^{3}H-methyl]methionine [the precursor of the methyl donor S-adenosyl-L-methionine (AdoMet)] followed by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) and alkaline hydrolysis of protein methyl esters showed that the peak of carboxyl methylation was in the 20- to 24-kD region of the gel, consistent with the molecular mass of $p21^{ras}$ and its homologs (Fig. 1A). Carboxyl methylation of these proteins was unaffected by cycloheximide and therefore independent of protein synthesis. An inhibitor of $p21^{ras}$ methylation (*7*), *N*-Acetyl-*S*-*trans*,*trans*-farnesyl-L-cysteine (AFC), inhibited carboxyl

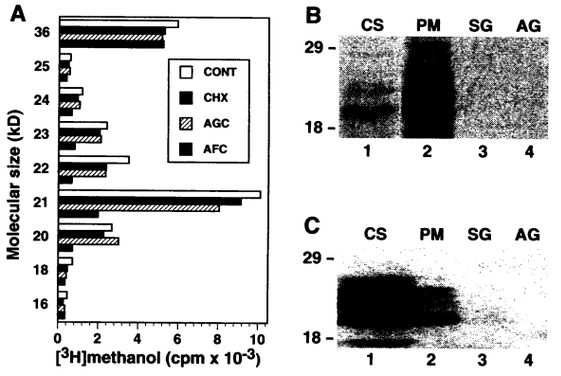

Fig. 1. Metabolic labeling of neutrophil Ras-related proteins. Human neutrophils were incubated with either [^{3}H-methyl]methionine (350 to 500 μCi/ml in Hepes-buffered salt solution for 1 hour) (A and B) or [5-^{3}H]mevalonolactone (115 μCi/ml in Hepes-buffered Dulbecco's modified Eagle's medium with 25 μM lovastatin) (C). (**A**) Trichloroacetic acid (TCA) precipitates of cells labeled with [^{3}H-methyl]methionine in the presence or absence (Cont., control) of either cycloheximide (CHX) (5 μg/ml), AGC (100 μM), or AFC (100 μM) were analyzed for carboxyl methylation by SDS-PAGE followed by alkaline hydrolysis of excised bands and quantitation of volatilized [^{3}H]methanol as described (*22*). As reported previously (*7*), the AFC-insensitive 36-kD carboxyl-methylated protein was not prenylated and is not associated with CAAX processing. Cells labeled with [^{3}H-methyl]methionine (**B**) or [5-^{3}H]mevalonolactone (**C**) were fractionated into cytosolic (CS), plasma membrane (PM), specific granule (SG), and azurophilic granule (AG) compartments as described (*23*) and analyzed by SDS-PAGE and fluorography. The 20- to 24-kD region of the gel shown in (B) was analyzed for carboxyl methylation as in (A). Of the total alkaline-labile counts recovered, 62% were from PM, 23% from CS, 12% from SG, and 3% from AG.

M. R. Philips, M. H. Pillinger, R. Staud, G. Weissmann, Division of Rheumatology, Department of Medicine, New York University Medical Center, New York, NY 10016.
C. Volker and J. B. Stock, Departments of Molecular Biology and Chemistry, Princeton University, Princeton, NJ 08540.
M. G. Rosenfeld, Department of Cell Biology, New York University Medical Center, New York, NY 10016.
*To whom correspondence should be addressed.
†Present address: Division of Rheumatology and Immunology, Department of Medicine, University of Florida, Gainesville, FL 32610.