ENRICHMENT OF SPECIFIC SUPPRESSOR T CELLS AND CHARACTERIZATION OF THEIR SURFACE MARKERS*

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T cells with suppressive properties may be distinguished from other T cells on the basis of their Ly and Ia antigen phenotypes (1). Antisera directed to I-J subregion gene products have been shown to eliminate suppressor T cells in functional assays (2). There has not, however, been a direct demonstration of the I-J product as a surface Ia antigen, probably because the proportion of suppressor T cells in lymphoid cell populations is very small. The aim of the present paper is to describe a method by which suppressor T cells may be enriched manyfold, and to characterize their surface markers.

Materials and Methods

Mice. 2- to 3-mo-old specific pathogen-free CBA/CaH Wehi mice were used.

Antigens. Human gamma globulin (HGG) (Cohn fraction II) was purchased from the Commonwealth Serum Laboratories, Melbourne, Australia. Deaggregated HGG was prepared according to the method of Basten et al. (3). Polymerized flagellin of Salmonella adelaide (Fla) was kindly supplied by Dr. J. Pye of the Walter and Eliza Hall Institute. The hapten dinitrophenyl (DNP) was purchased from Eastman Kodak Co., Rochester, N. Y., in the form of the sodium sulfonate salt. It was coupled to the proteins HGG and Fla, as described by Eisen (4), at the following substitution ratios: DNP-HGG, DNP-Fla. Keyhole limpet hemocyanin (KLH) was purchased from Calbiochem, San Diego, Calif., (catalog no. 374805) and bovine serum albumin (BSA) from Armour and Company Ltd., Eastbourne, England. For priming, mice were given 400 μg alum-precipitated HGG and 20 μg DNP-Fla (1, 3). For tolerization, mice received 2.5 mg deaggregated HGG intravenously. Spleens were used 4-8 wk after priming and 1-2 wk after tolerization (1, 3).

Cell Separation on Antigen or Antibody-Coated Dishes. Tissue culture grade polystyrene Petri dishes, 5 cm in diameter (Camelec Medical Products, Camden Park, South Australia) were coated with antibody or antigen according to the method of Mage et al. (5). In brief, they were incubated for 18-24 h at 4°C with 3 ml phosphate-buffered saline, and for a further 18-24 h with 3 ml antigen or antibody solution containing 1 mg/ml protein. The plates were washed five times with buffer and twice with Eisen’s balanced salt solution before spleen cells were added. Incubation with radioactively labeled HGG showed that under these conditions, 15-25 μg protein was bound and <0.1 μg was released during the subsequent removal of adherent spleen cells. To enrich for suppressor T cells, 2-3 ml of a suspension of 1.5 × 10⁶ spleen cells/ml was added to anti-mouse Ig-coated dishes. After incubation at room temperature for 1 h, the plates were swirled and the nonadherent cells were removed for further separation. 30% of the initial cell population was recovered at this stage and shown to contain over 95% of the Ig-spleen cells as detected by a fluorescein-labeled rabbit anti-mouse Ig reagent. 2.5 ml of this cell population, at a

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concentration of 10^7 cells/ml, was incubated at room temperature for 1 h in HGG-coated dishes. The nonadherent cells were then removed and the plates were allowed to rest on ice for 15 min. Under these conditions, the adherent cells were released and >90% were viable. This population was then investigated for surface characteristics and suppressive properties.

Antisera. Anti-Thy-1 serum was prepared and used as described before (1). Anti-Ly-1.1, anti-Ly-2.1, anti-Ia^a, and anti-Ia^k sera were kindly donated by Dr. I. F. C. McKenzie, Austin Hospital, Melbourne, Australia (1). Anti-I-J^k and anti-I-J^b sera were a generous gift of Dr. T. Tada, Chiba University, Chiba, Japan. For the anti-Ly sera, 10^6 cells/ml were exposed to a 1:4 or 1:6 dilution, and for the anti-Ia sera, 25 x 10^6 cells/ml were exposed to a 1:10 dilution. Rabbit serum served as a source of complement. Rabbit anti-mouse Ig or rabbit anti-HGG antibodies were purified by passing hyperimmune serum through mouse Ig- or HGG-coated columns and subsequently eluting using 0.15 M glycine HC1 buffer at pH 2.2.

Cytotoxic Assays. Cytotoxic assays were performed on ^51Cr-labeled spleen cells as described elsewhere (1).

Cell Transfers. The various spleen cell preparations were injected intravenously into irradiated recipients. These were then given 100 µg DNP-HGG intraperitoneally and their spleens were assayed 7 days later as described before (1, 3).

Plaque-Forming Cell (PFC) Assays. These assays were performed using DNP-Fab-coated sheep erythrocytes as described before (1).

Results
Spleen cells from CBA mice tolerized 7-14 days before with deaggregated HGG provide a source of suppressor T cells (3). When these cells were mixed with HGG-primed spleen cells and DNP-Fla-primed spleen cells, and given to irradiated mice challenged with DNP-HGG, suppression of the anti-DNP PFC response was obtained (Table I). When the HGG-tolerant spleen cells were fractionated first on rabbit anti-mouse, Ig-coated dishes and then on HGG-coated dishes, as detailed in Materials and Methods, the anti-DNP response was suppressed with as few as 3 x 10^6 cells. This degree of suppression was comparable to that obtained with 3 x 10^7 unfractionated spleen cells. There was no significant enrichment of cells with suppressive activities when further separation of Ig^-tolerant cells was performed with dishes coated with an irrelevant antigen (rabbit Ig) or with rabbit anti-HGG Ig (Table II).

The suppressive activity of the enriched suppressor cells from HGG-tolerant CBA mice was abrogated by pretreatment with anti-Thy-1, anti-Ia^a, anti-I-J^k, and anti-Ly-2.1 sera and complement, but was not affected by anti-Ia^k, anti-I-J^b, or anti-Ly-1.1 reagents (Table III). More than 30% of the cells of the enriched population could be killed directly by the appropriate anti-I-J serum in the presence of complement. These reagents had no effect on the nonadherent cell population (Fig. 1).

Discussion
The method of Mage et al. (5), which allows the purification of Ig^-cells from spleen, was successfully adapted to separate antigen-specific suppressor T cells. As determined by a functional assay, an enrichment of at least 100-fold was obtained by allowing the Ig^-fraction of HGG-tolerant spleen cells to adhere to HGG-coated dishes (Table I). The enrichment was specific since cells binding to an irrelevant antigen were not suppressive. Furthermore, the recovery of adherent cells was 8-10 times higher with HGG-coated dishes than with plates bearing other material (Table II). By contrast, the cells not adhering to the HGG-coated dishes were not suppressive (Table I). The results
### Table I

**Indirect Anti-DNP PFC in Irradiated Recipients of HGG-and DNP-Primed Spleen Cells**

| HGG-tolerant cells added | Number of tolerant cells given | Indirect anti-DNP-PFC per spleen (SE)* | Suppression |
|--------------------------|-------------------------------|---------------------------------------|------------|
| None given               | 116,470 (1.06)                |                                       |            |
| Unfractionated           | 3 x 10^4                     | 20,450 (1.16)                        | 82         |
| Nonadherent cells from rabbit anti-mouse Ig-coated dish | 3 x 10^4 | 4,410 (1.34) | 96 |
| HGG-coated dish          | 10^7 Nonadherent              | 118,270 (1.14)                       | 0          |
|                          | 3 x 10^6 Adherent             | 6,110 (1.29)                         | 95         |
|                          | 3 x 10^5 Adherent             | 6,100 (1.28)                         | 88         |

* Geometric means, SE, five mice per group.

### Table II

**Indirect Anti-DNP-PFC in Irradiated Recipients of HGG- and DNP-Primed Spleen Cells**

| Recovery of adherent cells as % total unfract. | Number of fractionated tolerant cells given to irradiated mice | Indirect anti-DNP PFC per spleen (SE)* | Suppression |
|-----------------------------------------------|---------------------------------------------------------------|---------------------------------------|------------|
| Neither                                      | 68,060 (1.15)                                                |                                       |            |
| Rabbit anti-mouse lg                         | 6,710 (1.45)                                                 | 90                                    |
| HGG (after anti-lg)                         | 4,110 (1.29)                                                 | 91                                    |
| HGG (after anti-lg)                         | 17,300 (1.37)                                                | 75                                    |
| Rabbit anti-HGG (after anti-lg)             | 6,000 (1.17)                                                 | 32                                    |
| BSA (after anti-lg)                         | 66,570 (1.21)                                                | 2                                     |
| Rabbit Ig (after anti-lg)                   | 60,370 (1.21)                                                | 11                                    |

* Geometric means, SE, five mice per group.

### Table III

**Characterization of Enriched Suppressor Cells**

| Treatment of fractionated HGG-tolerant cells | Indirect anti-DNP-PFC per spleen (SE)* |
|---------------------------------------------|---------------------------------------|
| Untreated                     | 96,220 (1.12)                         |
| Guinea pig complement (GPC)         | 21,160 (1.11)                         |
| Rabbit complement (RC)              | 14,640 (1.11)                         |
| Anti-Thy-1 + GPC                   | 14,200 (1.36)                         |
| Anti-Ia<sup>a</sup> + RC            | 119,500 (1.06)                        |
| Anti-Ia<sup>a</sup> + RC            | 85,450 (1.14)                         |
| Anti-I-J<sup>+</sup> + RC           | 17,250 (1.07)                         |
| Anti-I-J<sup>+</sup> + RC           | 72,090 (1.07)                         |
| Anti-I-J<sup>+</sup> + RC           | 21,130 (1.05)                         |
| Anti-Ly-1:1 + RC                   | 23,370 (1.49)                         |
| Anti-Ly-2:1 + RC                   | 73,960 (1.14)                         |

* Geometric means, SE, five mice per group.

Anti-I<sup>j</sup> represents A.TH anti-A.TL, anti-I<sup>a</sup> A.TL anti-A.TH, anti-I<sup>j</sup> B10.A(3R), anti-I<sup>j</sup> B10.A(3R); anti-I<sup>a</sup> A.TH, anti-I<sup>j</sup> B10.A(3R), anti-I<sup>a</sup> A.TH.

are in agreement with those of Maoz et al. (6) who fractionated antigen-specific suppressor and helper cells on antigen-derivatized collagen gel-coated plates and observed specific binding of 1–2% of the cells; nonspecific binding being <0.5%.
FIG. 1. Effect of anti-Ia\(^k\) (O), anti-I-J\(^k\) (△), anti-Ia\(^\prime\) (●) and anti-I-J\(^{\prime}\) (▲) sera and complement on fractionated spleen cells from HGG-tolerant mice. (A) effect on HGG-binding cells; (B) effect on non-HGG-binding cells. All cells were incubated for 45 min at 45°C after treatment with complement to release their chromium. The background in these experiments was <20%.

The enriched suppressor cells were Ig\(^-\) and Thy-1\(^+\), and therefore had the characteristic features of T lymphocytes. In addition, they were susceptible to anti-Ly-2.1, not to anti-Ly-1.1 serum, indicating that they belonged to the Ly-2,3 subset, as had been demonstrated before with unseparated cells (1). As shown in Table III, the enriched cells were sensitive to anti-Ia serum, and more specifically, to an antiserum specific for the I-J subregion gene product. They must therefore carry the Ia-4 determinant defined by the I-J subregion, a determinant also shown to be present on allotype (2) and carrier-primed (7) suppressor T cells. Finally, we were able to demonstrate directly the presence of the I-J subregion gene product as a cell surface component, since ~30% of the enriched cells, but none of the nonadherent cells, were killed by anti-I-J\(^{\prime}\) serum and complement. These results are in agreement with data recently obtained by Okumura et al. (8) who enriched specific suppressor T cells from primed mice by adsorption to, and elution from antigen-coated Sephadex G-200 columns.

As HGG was used to coat dishes in our system, cells with Fc receptors (FcR) could have been removed along with HGG-specific cells. Furthermore, if suppressor cells bear FcR, their enrichment may not have been antigen-dependent, but rather FcR-dependent. We feel that this is unlikely for three reasons. First, the incubation in HGG-coated dishes was preceded by one in dishes coated with rabbit Ig (anti-mouse Ig) during which most FcR\(^-\) cells should have been removed. Secondly, recent functional studies on I-J\(^{\prime}\) suppressor T cells indicated that these belonged to an FcR\(^-\) subset (8). Thirdly, it has been shown that the majority of FcR\(^+\) T cells expressed Ia antigens controlled by genes in the I-A, I-C, S, and G regions of the major histocompatibility complex and that determinants controlled by loci in the I-J and I-E subregions were not detectable on FcR\(^+\) cells (9). Taken together, these points argue against the possibility that our enrichment procedure is FcR-dependent. Some FcR\(^+\) cells could, however, have escaped the first dish and might thus contami-
nate our final population. They may indeed account for those non-I-J-bearing cells which were susceptible to anti-Ia sera (Fig. 1).

The enrichment procedure described here should facilitate a variety of studies; for example, elucidation of the interactions between suppressor T cells and their targets, identification of factors with suppressive properties, and production of suppressor T-cell lines by cell hybridization techniques.

Summary

A simple method is described which allows antigen-specific suppressor T cells to be enriched by >100-fold. The enriched cells have the following characteristic markers: Ig−, Thy-1+, Ly-1−, Ly-2,3+, and I-J+. More than 30% of this population could be killed directly by an antiserum specific for the I-J subregion gene product in the presence of complement.

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