# Commercial human albumin preparations for clinical use are immunosuppressive *in vitro*

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***Objective:*** **We previously reported significant variations in oxidation status and molecular length among sources and lots of human serum albumin (HSA) commercial preparations intended for clinical use. In this report, we investigated what effect the presence of HSA products have on the immune response *in vitro*.**

***Design:*** **Laboratory study.**

***Setting:*** **Trauma research basic science laboratory.**

***Subjects:*** **Activated human peripheral blood mononuclear cells.**

***Interventions:*** **Six commercial HSA preparations were tested for their effect on cytokine release from activated human peripheral blood mononuclear cells (PBMCs) and T-lymphocytes. Mass spectrometry analysis of aspartyl-alanyl diketopiperazine (DA-DKP) content of HSA and percentage of HSA having lost its amino terminal dipeptide aspartyl alanyl (HSA-DA) were correlated.**

***Measurements and Main Results:*** **Human PBMCs were cultured in the presence of six commercial HSA preparations and activated via the T-cell receptor complex. A cloned T-lymphocyte cell line, activated with specific antigen, was also cultured with both synthetic DA-DKP and small molecular weight extracts from the commercial HSA tested. Supernatants were quantified by enzyme-linked immunosorbent assay for interferon-$\gamma$ and tumor necrosis factor-$\alpha$ content. DA-DKP was extracted from HSA by centrifugal filters and quantified by anion exchange liquid chromatography coupled to negative electrospray ionization mass spectrometry. HSA species were determined by reverse phase liquid chromatography coupled to positive electrospray ionization, time of flight mass spectrometry. All HSA preparations significantly inhibited the *in vitro* production of interferon-$\gamma$ and tumor necrosis factor-$\alpha$ by activated PBMCs. DA-DKP was detected in all HSA sources at concentrations ranging between 42.0 and 79.6 $\mu$M. A synthetic form of DA-DKP possessed similar immunosuppressive qualities in a dose-dependent manner on T lymphocytes.**

***Conclusions:*** **DA-DKP was present in significant concentrations in all HSA sources tested and was partially responsible for the immunosuppressive effects of HSA on activated PBMCs and T-lymphocytes *in vitro*. In view of these findings, administering HSA to immunocompromised critically ill patients might be reevaluated. (Crit Care Med 2006; 34:1707–1712)**

**Key Words: human serum albumin; peripheral blood mononuclear cells; T-lymphocyte; cytokine; diketopiperazine**

Conflicting results on the effects of albumin administration to critically ill patients have been reported (1–8). Commercially available human serum albumin (HSA) products contain significant variability in post-translational species of HSA (i.e., cysteinylation, nitrosylation, truncation, etc.), native HSA content, and degradation products (9). We were interested to determine what effect these modifications to albumin might have on the immune response of patients receiving HSA products. Since albumin is often administered to patients who might be immunosuppressed following traumatic injury, we evaluated the effect of HSA product on the production of proinflammatory cytokines.

Six commercial HSA preparations were tested for their effect on the *in vitro* production of interferon (IFN)-$\gamma$ and tumor necrosis factor (TNF)-$\alpha$ by activated peripheral blood mononuclear cells (PBMCs) and T lymphocytes. We found that at amounts calculated to be present *in vivo*, all six preparations significantly inhibited cytokine production from PBMCs and T lymphocytes. The factors responsible for the observed immunosuppressive activity could be removed by dialysis of the less than 12,000 molecular weight (MW) material and was found to be present in the less than 3000 MW fraction.

One constituent responsible in part for the immunosuppressive activity was identified as aspartyl-alanyl diketopiperazine (DA-DKP) by liquid chromatography coupled to mass spectrometry. Diketopiperazines in biological preparations are degradation and cyclization products of the N termini of proteins and peptides (10–12). Albumin is a 585 amino acid plasma protein containing aspartic acid as the N terminal amino acid followed by alanine. Chan et al. (13) previously reported the formation of a diketopiperazine from albumin on extreme and prolonged heating.

These results demonstrate that factors present in commercial HSA products may affect T-cell receptor signal transduction pathways that normally result in the activation of transcription factors and cytokine production. The implications of these findings and the use of HSA in treating critically ill patients are discussed.

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## MATERIALS AND METHODS

*HSA Preparations.* Six commercially available 25% HSA solutions (Alpha Therapeutic, Los Angeles, CA; Aventis Behring, Kankakee, IL; Bayer Corporation, Elkhart, IN; Baxter Healthcare, Westlake Village, CA; Grifols Biologicals, Barcelona, Spain; ZLB Bioplasma, Berne, Switzerland) were evaluated for effect on *in vitro* cytokine production by activated human PBMCs and a T-lymphocyte cell line.

This study was approved by the HealthONE Alliance Institutional Review Board according to guidelines published by the HHS Office for Protection from Research Risk.

*Cell Culture.* Venous blood was collected by venipuncture from three different healthy human donors (male and female). The mononuclear fraction of peripheral blood leukocytes was collected by centrifugation on Histopaque (Sigma Chemical, St. Louis, MO) and washed in Iscoves modified Dulbecco's minimal essential medium (IMDM, American Type Culture Collection, Rockville, MD). A cloned human T-lymphocyte cell line that produces cytokine in response to antigen presented by HLA-DR autologous cells was also used for this study.

Stimulation of cells in culture was performed as follows. First, $2 \times 10^5$ human PBMCs were precultured in the presence of the indicated HSA preparations at a final *in vitro* concentration of 5% in IMDM plus 10% heat inactivated autologous serum for 1 hr at 37°C. Anti-CD3/anti-CD28 beads (Dynal, Oslo, Norway) were then added at predetermined optimal concentrations to activate T lymphocytes. For the cloned T-lymphocyte line, 0.1% glutaraldehyde-fixed Epstein Barr virus-transformed autologous peripheral blood leukocytes as antigen presenting cells and 25 μg/mL influenza A hemaglutinin peptide 307–319 (as antigen) in serum-free IMDM were added to effect near-optimal cellular activation (75% maximal) as evidenced by cytokine production in prior studies. All cultures were incubated overnight at 37°C, and an aliquot of the supernatant was then harvested for quantification by enzyme-linked immunosorbent assay (ELISA).

*Cytokine Quantification.* ELISAs for IFN-γ (BioSource, Camarillo, CA) and TNF-α (Pierce Endogen, Rockford, IL) were performed according to the manufacturer's instructions. The sensitivity of the assays detecting IFN-γ averaged 10 pg/mL and for TNFα was 4 pg/mL.

*HSA Fractionation.* Dialysis of the commercial HSA product used a 12,000 MW cutoff membrane against water for 72 hrs, followed by equilibration with phosphate-buffered saline, and removed approximately 90% of <15,000-Da MW material by liquid chromatography-mass spectrometry. The concentrations of dialyzed HSA product were adjusted to equal that of the starting material by centrifugal concentration (Vivascience, Edgewood, NY).

The less than 3000 MW components of the HSA products were obtained using centrifugal concentrators (Vivascience, Edgewood, NY). Volumes of the filtrates were adjusted to equal that of the starting material using phosphate-buffered saline.

*DA-DKP Analysis.* Fifty microliters of each of the <3000-Da fractions of commercial albumin were injected into high-performance liquid chromatography (HPLC, 2795 system, Waters, MA) coupled to a mass spectrometer (LCT-TOF, Micromass, UK), and DA-DKP was quantified using a strong anion exchange column (Supelcosil, SAX1 250 mm × 4.6 mm, Supelco) and a 70:30 v/v methanol/water with 2 mM ammonium acetate (Sigma Aldrich, St. Louis, MO) as the mobile phase in an isocratic mode at 1 mL/min. The output of the HPLC was split 1:20 (v/v) and injected into the mass spectrometer using negative electrospray ionization (−ESI MS) with a scan range of 80–1000 m/z, cone voltage of 30 eV, source temperature of 100°C, and gas temperature of 250°C. DA-DKP was measured by monitoring the mass 185 in time which corresponds to DA-DKP minus a single proton ($-H^+$). DA-DKP elutes at 5.8 mins and is quantified by integrating the area under the curve. The area was compared with a standard curve derived from synthetic DA-DKP standards (DMI Synthesis, Newport, Wales) of known concentrations (5000 ng/mL, 1000 ng/mL, 200 ng/mL, 40 ng/mL, 8 ng/mL). The calibration curve was found to be very linear in this range with an $R^2$ of .99998.

*Albumin Analysis.* HSA products were analyzed by HPLC coupled to positive electrospray ionization time of flight mass spectrometry (+ESI MS TOF) as previously described (9). Briefly, each HSA formulation (25% w/v) was diluted 1:10 with $dH_2O$. Then 10 μL of each sample was injected onto a YMC-Pack Protein-RP, 150 mm × 4.6 mm, 5-μ, HPLC column heated to 50°C (Waters, Milford, MA) using a 20-min linear gradient method using water/0.1% trifluoroacetic acid (A) and acetonitrile/0.1% TFA (B) at 1 mL/min.

The output of the HPLC was split 1:20 (v/v) and injected into the mass spectrometer with a scan range of 500–3500 m/z, cone voltage of 30 eV, source temperature of 100°C, and gas temperature of 250°C. Albumin elutes at 8.15 mins and is visualized as a charge envelope from 1100 to 2500 m/z representing +44 to +26 charges. The spectrum was then decon-

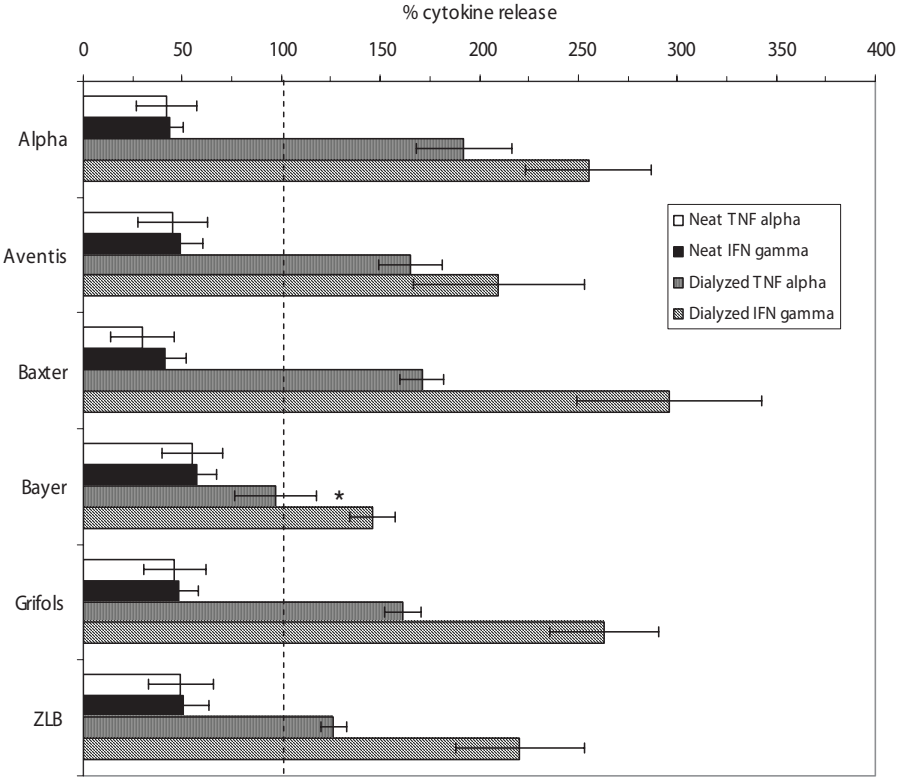


**Figure 1.** Tumor necrosis factor (*TNF*)-α and interferon (*IFN*)-γ *in vitro* cytokine production by human donor peripheral blood mononuclear cells (PBMCs) in the presence of neat (unaltered product as obtained from manufacturer) and dialyzed commercial human serum albumin products. Shown are the averaged results from three experiments using three different donors PBMCs, male and female, with indicated standard error. Dotted line represents cytokine measured in cultures stimulated with CD3/28 beads in the presence of control compounds (20 mM N-acetyl-tryptophan for neat product and phosphate-buffered saline for dialyzed). Bars left of the line indicate a reduction in cytokine, whereas bars extending past to the right, an increase. All differences were significant ($p < .05$) except *$p > .05$, not significant vs. 100% release. Positive control cultures (PBMCs plus anti-CD3/CD28) varied from 425 to 776 pg/mL TNF-α and from 100 to 940 pg/mL IFN-γ, depending on the donor.

volved to the uncharged parent mass using MaxEnt 1 (Micromass, UK). The parent mass spectrum was then integrated, and relative proportions of each species were calculated.

*Statistical Analysis.* All cytokine assays were performed in triplicate determinations and results shown as mean with standard error. We calculated *p* values in Microsoft Excel using Student's *t*-test. Correlation coefficients were calculated using linear regression in Excel. The significance level was set at .05.

## RESULTS

Commercial HSA products inhibited the *in vitro* production of IFN-γ and TNF-α by human PBMC in response to stimulation through the T-cell receptor complex (Fig. 1). This immunosuppressive activity was consistently demonstrated for both cytokines irrespective of the age or gender of the PBMC donor. Included as a control for these experiments was 20 mM N-acetyl-tryptophan (NAT), which was present in each of the commercial HSA products. There were no significant differences in cytokine levels in cultures supplemented with 20 mM NAT compared with CD3/28 stimulation cultures without NAT. The presence of commercial HSA led to the reduction of IFN-γ and TNF-α by an average of 52 ± 3.3% and 55 ± 4.9%, respectively. The Baxter product was the most effective tested, exhibiting a reduction of 59 ± 16.3% for IFN-γ and 70 ± 11.1% for TNF-α. Bayer proved to be the least immunosuppressive, with 43 ± 9.9% and 45 ± 15.6% reductions in cytokine, respectively. Cytokines detected in cultures not stimulated with anti-CD3 plus anti-CD28 were always <35 pg/mL for IFN-γ and 41 pg/mL for TNF-α.

The removal of low-MW components from HSA was achieved by extensive dialysis using a 12,000-Da MW cutoff membrane and confirmed by −ESI MS. Treatment of PBMC with the dialyzed HSA products tested caused a significant increase in cytokine production following stimulation in all but one of the products tested, the exception being Bayer and TNF-α (Fig. 1). *In vitro* CD3/CD28-induced IFN-γ and TNF-α were increased by an average of 232 ± 30.1% and 152 ± 19.8% respectively (TNF-α mean includes the Bayer value). The most dramatic stimulatory effect was observed after treatment with the dialyzed Baxter product, resulting in a 295 ± 47% increase in IFN-γ.

Since dialysis removed the inhibitory components of the commercial HSA products, we collected by centrifugation the low-MW constituents using centrifugal concentrators having 3000 MW cutoff membranes. Due to the nature of anti-CD3 plus anti-CD28 stimulation of human PBMCs, the cytokine producing cells in the previous experiments were predicted to be T lymphocytes. To exclude the possibility of an effect on other cytokine-producing populations present in PBMCs, we used a T-lymphocyte cell line stimulated *in vitro* using specific antigen and autologous glutaraldehyde-fixed presenting cells to test the low-MW fractions effect on IFN-γ and TNF-α production. Pretreatment of the influenza hemaglutinin-specific TRiPS T-lymphocyte cell line with the <3000 MW fractions of the commercial albumin products caused a decrease in TNF-α production for all preparations tested (Fig. 2) by a mean of 37 ± 4.8% (36 ± 9.6% decrease in IFN-γ) with the Bayer product effecting a maximal reduction of cytokine by 46%. Only the alpha product effect on cytokine production was not statistically significant (30% inhibition, $p = .089$); all other <3000 MW fractions significantly inhibited T-lymphocyte production of cytokine (Fig. 2).

Comparison of the mass spectrometry profiles of HSA obtained from plasma of a healthy donor vs. the commercial HSA products showed several important differences. In all six of the commercial HSAs, truncation was observed at both the N- and C-termini of the albumin molecule, as well as cysteinylation, glycation and nitrosylation (Fig. 3).

Previous work from this laboratory has identified the presence of a diketopiperazine formed by the cyclization of the two N-terminal amino acids of human albumin, aspartate and alanine (DA-DKP) in human plasma. DA-DKP was detected in and quantified by mass spectrometry from all six commercial HSA products (Table 1).

Linear regression analysis indicated that the amount of DA-DKP present in the commercial HSA products correlated with the percentage of albumin species missing the two N-terminal amino acids with an correlation coefficient ($R^2$) of .796 ($p < .001$). Mass spectrometry analysis confirmed the absence of DA-DKP from the dialyzed HSA preparations that did not have *in vitro* immunosuppressive activity.

These data suggested that DA-DKP is present in the small molecular weight components of the commercial HSA products in sufficient quantity (μM amounts) to potentially affect T-lymphocyte activation. When tested, synthetic DA-DKP inhibited the antigen-induced *in vitro* TNF-α production by the T-lymphocyte cell line in a dose-dependent manner (Fig. 4). In the presence of 50–100 μM

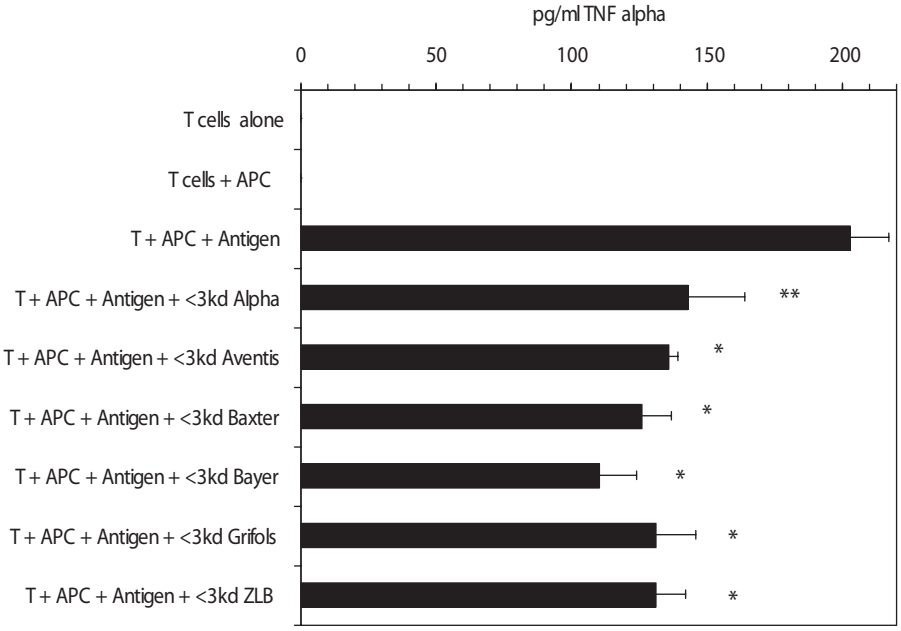


**Figure 2.** Inhibition of *in vitro* tumor necrosis factor (*TNF*)-α production from a cloned antigen-specific T-lymphocyte cell line activated by specific antigen plus autologous antigen presenting cells (*APC*) in the presence of <3000 molecular weight filtrates recovered from commercial human serum albumin products. Shown are the results from a single representative experiment performed as triplicate determinations with indicated standard error (*$p < .05$, **$p = .089$).

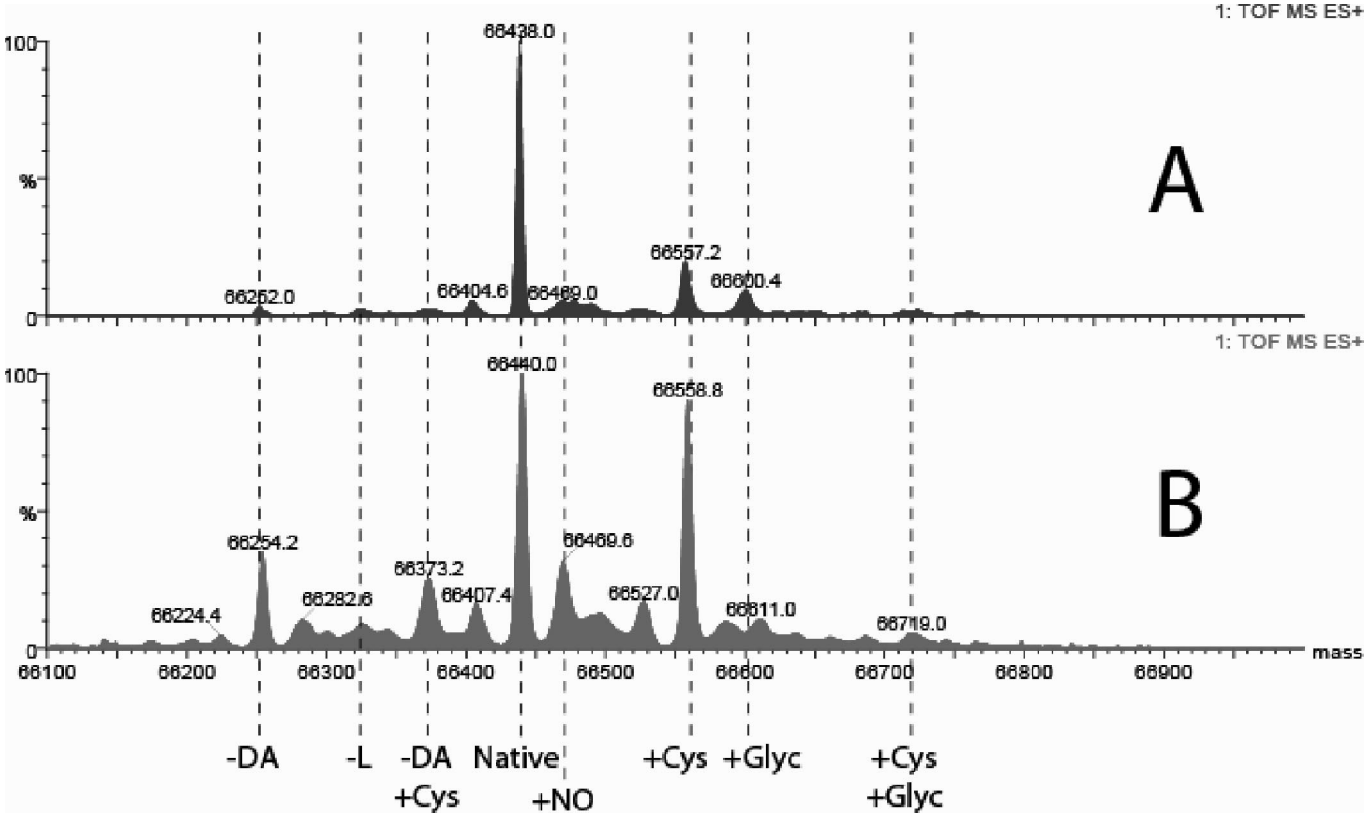


**Figure 3.** *A*, normal healthy volunteer human serum albumin (HSA) profile. *B*, typical commercial albumin preparation. HSA identification: *−DA*, aspartate-alanine absent from N-terminus; *−L*, leucine absent from C-terminus; *+Cys*, cysteine 34 is bound by a free cysteine; *+Glyc*, glycated albumin; *+NO*, nitrosylation.

**Table 1.** Quantification by mass spectrometry of aspartyl-alanyl diketopiperazine (DA-DKP) and % of all human serum albumin (HSA) having lost its amino terminal dipeptide aspartyl alanyl (HSA-DA) species in commercial HSA products (in triplicates)

| | DADKP μM (Average) | %HSA-DA |
|---|---|---|
| Alpha | 60.2 ± 7.9 | 16.3 ± 3.1 |
| Aventis | 79.6 ± 7.0 | 24.4 ± 1.5 |
| Baxter | 42.0 ± 4.1 | 11.3 ± 5.2 |
| Bayer | 59.9 ± 2.3 | 10.7 ± 5.7 |
| Grifols | 58.6 ± 2.5 | 12.4 ± 2.7 |
| ZLB | 75.4 ± 10.3 | 16.6 ± 3.0 |

DA-DKP, an amount similar to that quantified by mass spectrometry in the commercial HSA products (Table 1), cytokine production by T-lymphocytes was decreased by 43 and 52%, respectively, compared with the positive control. This decrease in cytokine production was similar in magnitude to that observed with CD3/CD28-activated human PBMC in the presence of the commercial HSA products (Fig. 1).

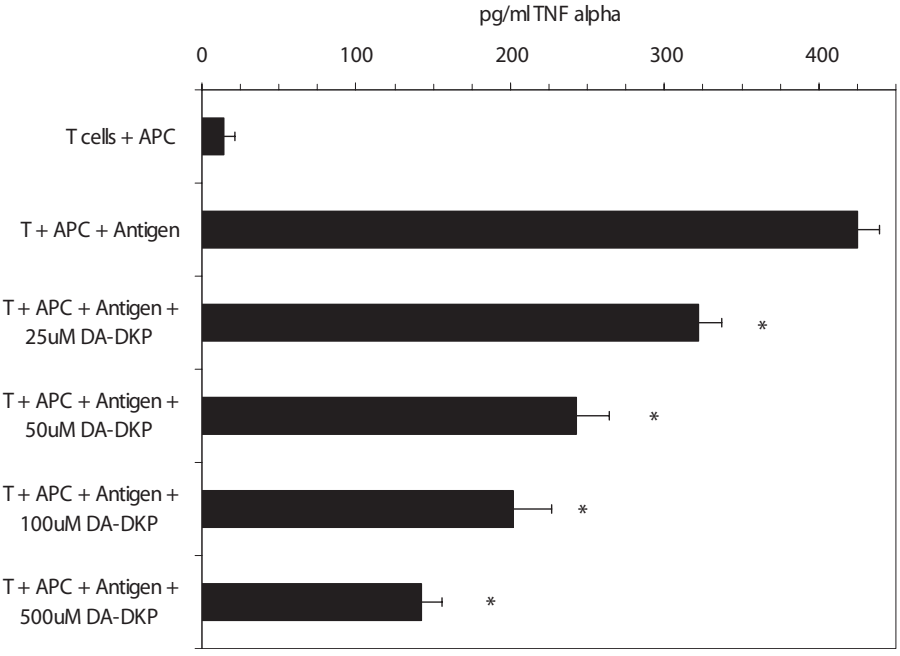


**Figure 4.** Inhibition of tumor necrosis factor (*TNF*)-α production from a cloned antigen-specific T-lymphocyte cell line activated by specific antigen plus autologous antigen presenting cells (*APC*) in the presence of synthetic aspartyl-alanyl diketopiperazine (*DA-DKP*). Shown are the results from a single representative experiment performed as triplicate determinations with indicated standard error (*$p < .05$).

## DISCUSSION

We have identified an immunosuppressive activity in six commercial HSA products intended for clinical use. Addition of the HSA product at concentrations similar to that used in clinical practice to *in vitro* cultures of activated human PBMCs or a cloned antigen-specific T-lymphocyte cell line inhibited the production of the immuno-inflammatory cytokines IFN-γ and TNF-α. Dialysis of the products to remove the low-MW fractions resulted not only in abrogation of the immunosuppressive effect but an enhanced cytokine production. We cannot explain, at this time, if the increased cytokine production we observed is due to any modifications inherent to the nondialyzable (large molecular weight) albumin or a nonspecific chaperoning effect that albumin has been speculated to possess.

The *in vitro* immunosuppressive activity of the HSA products could be recovered by fractionation of the low-MW components (≤3000 Da) by centrifugal concentration. The activity of these fractions was not as potent as the original HSA preparations, most likely due to the inefficiency of the centrifugal filtration methodology. However, the small MW fractions consistently reduced cytokine production by both human PBMCs and the human T-lymphocyte cell line, suggesting that the inhibitors were of small MW in nature. We included controls for an individual component added as a stabilizer to the HSA products, N-acetyltryptophan (present at 20 mM in the reconstituted HSA products). However, we could not identify all of the small MW components detected in the HSA products or determine the individual contribution of those unidentifiable components to the observed inhibition of *in vitro* cytokine production.

The *in vitro* immunosuppression could be at least partially attributed to a component found in all six commercial HSA products tested, DA-DKP. The commercial 25% HSA used in these experiments contained 42.0–79.6 μM of DA-DKP as determined by −ESI MS. The relatively high concentrations of this cyclic dipeptide originate from the N-terminal dipeptide aspartate alanine of HSA. DA-DKP concentration correlated in a linear fashion to the truncated HSA-DA. Thermal cyclization of both dipeptides and proteins (such as HSA) to form diketopiperazines has been documented in the literature (13–16). Heating a solution of HSA (part of the manufacturing process of HSA is to heat at 60°C for 10 hrs) has been shown to produce DA-DKP (a sevenfold increase in DA-DKP upon heating at 60°C for 10 days, our unpublished observation). The cleavage and cyclization of aspartate-alanine (Fig. 5) is initiated by a nucleophilic attack of the aspartic acid amino terminal nitrogen's electrons lone pair on the alanine-histidine carbonyl of the amide bond, facilitating ring closure and DA-DKP formation (17). The rate of formation of DA-DKP is controlled, mainly, by temperature. HSA-DA does not undergo further cyclization of the next dipeptide because the higher temperatures needed to produce histidyl-lysyl diketopiperazine will inevitably cause denaturation of the truncated HSA before the possible diketopiperazine formation.

In these experiments, we did not detect any increase in T-lymphocyte production of cytokines considered immunosuppressive in nature (the TRiPS T-lymphocyte cell line can produce interleukin [IL]-4, IL-10 and IL-13 following activation). In addition, the release of preformed cytokine, IL-8 and IL-16, from the antigen-stimulated T-lymphocyte cell line was not inhibited in these experiments. Preformed cytokine is released from intracellular depots upon cell stimulation, and its presence *in vitro* is not dependent on activation of messenger RNA transcription. Together these observations suggest that the immunosuppressive effect of the commercial HSA products and DA-DKP is to suppress cytokine production at the transcriptional level. A preliminary molecular analysis suggests that a transcriptional factor secondary to T-cell receptor activation is inhibited that down-regulates all cytokine gene transcription in treated cells.

Therapeutic indications for the administration of HSA include the emergency treatment of shock, urgent restoration of blood volume, acute management of burns, clinical conditions associated with hypoproteinemia (18), and treatment of patients with acute lung injury (1–2). Considering the conflicting data concerning the effects of albumin administration to critically ill patients (1–8), our previously reported (9) heterogeneity in its composition, and the data presented here suggesting immunosuppressive activity, the clinical administration of HSA preparations to critically ill patients merits caution.

Definition of the composition of HSA used in critically ill patients, with particular attention to the oxidized forms of albumin and small MW compounds such as DA-DKP, is fundamental to an understanding of any therapeutic effect. The observed heterogeneity in composition could potentially influence clinical outcome and should be correlated to morbidity/mortality in randomized trials (19). Our data might possibly suggest that the immune status of the patient and possible immunosuppressive consequences be considered before any use of commercial HSA products. Additionally,

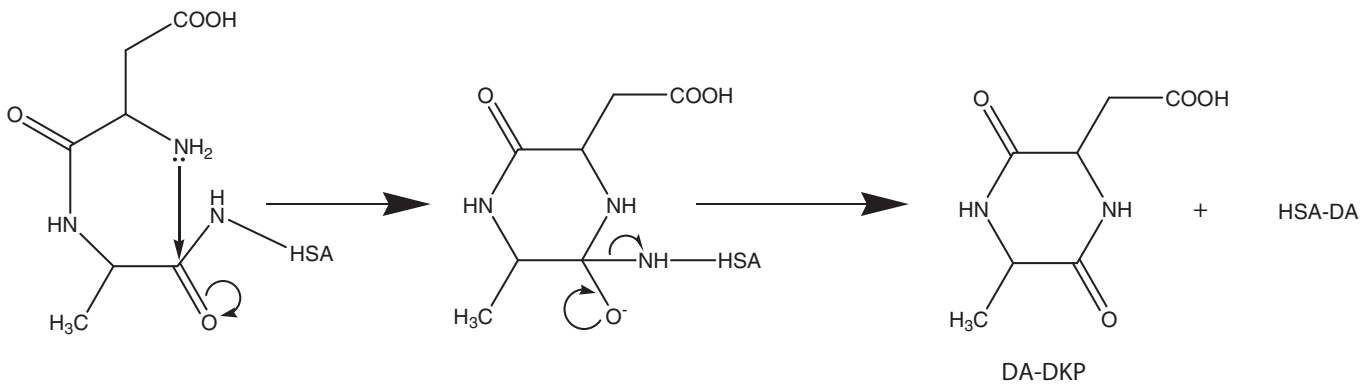


**Figure 5.** The cleavage from human serum albumin (*HSA*) and cyclization of aspartate-alanine (*DA*). *DA-DKP*, aspartyl-alanyl diketopiperazine.

all *in vitro* studies using research grade, biologically derived HSA without attention to its oxidation state, small MW constituents, and other posttranslational modifications should be viewed with caution.

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