Inhibition of Human Helper T Cell Function In Vitro by D-Penicillamine and $CuSO_4$

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ABSTRACT The effect of D-penicillamine (Pen) and mixtures of Pen and copper sulfate on the capacity of normal human peripheral blood mononuclear cells (PBM) to generate immunoglobulin-secreting cells (ISC) in response to the T-cell-dependent polyclonal B-cell activators pokeweed mitogen (PWM) and staphylococcal protein A (SPA) was examined. PBM obtained from normal individuals were incubated for 1–2 h at 37°C with medium alone, Pen, $CuSO_4$, or a mixture of Pen and $CuSO_4$. After washing, the cells were incubated for 6–7 d with PWM or SPA and then, with a reverse hemolytic plaque assay, assayed for the number of ISC generated. Preincubation of PBM with either Pen (100 μg/ml) or $CuSO_4$ (2 μg/ml) did not alter the subsequent capacity of the cells to generate ISC in response to PWM or SPA. In contrast, responsiveness to both mitogens was nearly abolished when PBM were similarly preincubated with a mixture of Pen and $CuSO_4$. Inhibition of responsiveness could not be ascribed to cell death, carry-over of the inhibitors, or an alteration in the concentration of PWM or the length of incubation yielding maximum responses. Co-culture experiments demonstrated that Pen and $CuSO_4$ preincubation had not caused augmented suppressor cell function. Experiments in which PBM were separated into adherent and nonadherent populations indicated that Pen and $CuSO_4$ preincubation inhibited the responsiveness of the nonadherent cells but did not alter the accessory cell function of monocytes. To determine whether Pen and $CuSO_4$ preincubation effected T- or B-cell function, PBM were separated into B- and T-cell-enriched populations, individually preincubated with Pen and $CuSO_4$, and then co-cultured with PWM. The results indicated that Pen and $CuSO_4$ markedly inhibited helper T-cell function and had little effect on the capacity of B cells to generate ISC. The observation that in the presence of $CuSO_4$ Pen inhibits helper T-cell activity may, in part, explain the therapeutic efficacy of Pen in rheumatoid arthritis and especially the capacity of Pen therapy to decrease antiglobulin titers in treated patients.

INTRODUCTION

D-Penicillamine (Pen)[1] has been demonstrated to be an effective agent in the treatment of rheumatoid arthritis (1–5). Despite extensive clinical experience with this drug, the explanation for its capacity to suppress rheumatoid inflammation remains unclear. A number of potential mechanisms for the action of Pen have been suggested, including the possibility that it might dissociate immunoglobulin (Ig)M antiglobulins in vivo (6–9), interfere with collagen cross-linking in synovial structures (10–14), exert an antiinflammatory action (15–20), or interfere with polymorphonuclear leukocyte chemotaxis (21–23). However, none of these postulated modes of action has been convincingly demonstrated to pertain in either experimental animals or treated patients.

An alternate explanation for the mechanism of action of Pen in rheumatoid arthritis is suggested by the clinical observations that therapy with Pen frequently results in lowered antiglobulin titers (1, 9, 24, 25), decreased levels of circulating immune complexes (26–28), and, often, diminished levels of serum immunoglobulins (27–30). This suggests the possibility that Pen might exert an immunosuppressive action and, thus, slow the progress of rheumatoid arthritis by sup-

Presented in part at the Annual Scientific Meeting of the American Rheumatism Association, Denver, Colorado, 31 May 1979.

Dr. Lipsky is a recipient of a National Institutes of Health Research and Career Development Award, 1-KO4-AM00599.

Dr. Ziff is a recipient of a U. S. Public Health Service Research Career Award.

Received for publication 4 December 1979 and in revised form 16 January 1980.

[1]*Abbreviations used in this paper:* HBSS, Hanks' balanced salt solution; ISC, immunoglobulin-secreting cells; Mϕ, monocytes; NAC, nonadherent cells; N-SRBC, neuraminidase-treated sheep erythrocytes; PBM, peripheral blood mononuclear cells; PEN, D-penicillamine; PWM, pokeweed mitogen; SPA, staphylococcal protein A.

pressing the ongoing immunological processes that underlie the chronic inflammation. Support for this idea comes from the studies of Hunneyball et al. (31) who found that rabbits given 15 mg/kg of Pen orally exhibited a depressed in vivo antibody response to immunization with egg albumin.

By examining the effect of Pen on responsiveness of human lymphocytes in vitro, we have explored the possibility that Pen may exert an immunosuppressive influence. Initial studies indicated that a brief exposure of human peripheral blood mononuclear cells (PBM) to Pen in the presence of copper ions inhibited mitogen-induced T-cell proliferation (32). In the current studies, the effect of a similar preincubation with Pen and $CuSO_4$ on the capacity of human PBM to generate immunoglobulin-secreting cells in response to stimulation with polyclonal B-cell activators has been examined. The data indicate that the mixture of Pen and $CuSO_4$ inhibits responsiveness by selectively inhibiting helper T-cell activity. These observations support the idea that Pen has an immunosuppressive action and may help to explain its action in patients with rheumatoid arthritis.

METHODS

Cell preparation. PBM were obtained from normal adult volunteers by centrifugation of heparinized venous blood on sodium diatrizoate/Ficoll cushions (Isolymph, Gallard-Schlesinger Chemical Mfg. Corp., Carle Place, N. Y.) as previously described (33). The cells were washed three times in Hanks' balanced salt solution (HBSS) before culture or further processing.

Reagents. Pen was obtained from Merck Sharp & Dohme Div., West Point, Pa. Pokeweed mitogen (PWM, lot A665710) was purchased from Grand Island Biological Co., Grand Island, N. Y., and *Staphylococcus aureus* protein A (SPA) from Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, N. J.

Culture medium. All cultures were carried out in RPMI 1640 medium (Microbiological Associates, Walkersville, Md.), supplemented with penicillin G (200 U/ml), gentamicin (10 μg/ml), L-glutamine (0.3 mg/ml), and 10% fetal bovine serum (Microbiological Associates).

Preincubation. Cells were suspended in serum-free culture medium at a concentration of about 5×10^6 cells/ml. These were incubated on a rotator at 37°C with Pen, $CuSO_4$, or a mixture of Pen and $CuSO_4$ at various final concentrations. Routinely, preincubations of 1–2 h were used. Control cells were preincubated in a similar manner in medium alone. At the end of the preincubation period, the cells were washed three times with HBSS and resuspended in fresh medium containing 10% fetal bovine serum for culture.

Measurement of cell viability. Cell viability was estimated using a combination of ethidium bromide (34) and fluorescein diacetate (35). Cell suspensions were incubated with the fluorescent probes (2 and 5 μg/ml, respectively) for 10 min at room temperature and the number of viable cells quantitated with a fluorescence microscope.

Cell separation. PBM were incubated on glass petri dishes as previously described (33, 36) to separate them into populations of adherent and nonadherent cells (NAC). NAC were harvested, incubated on a second petri dish to remove residual adherent cells, and then decanted and suspended in fresh medium for culture. Adherent cells were harvested from the initial petri dish with a rubber policeman, treated with mitomycin-C (40 μg/ml, Sigma Chemical Co., St. Louis, Mo.) for 45 min at 37°C, washed extensively, and suspended in fresh medium for culture. The number of monocytes (Mϕ) in each population was determined by staining for nonspecific esterase activity using α-naphthyl butyrate as a substrate (37) and by estimating the number of cells capable of ingesting latex particles. NAC contained <1% Mϕ, whereas 85–90% of the adherent-cell population was Mϕ.

In some experiments, NAC were separated into T- and B-cell-enriched populations by rosetting with neuraminidase-treated sheep erythrocytes (N-SRBC) (38), followed by centrifugation on diatrizoate/Ficoll cushions. The interface cells were harvested and again rosetted with N-SRBC to remove residual T cells. After centrifugation on diatrizoate/Ficoll cushions, the interface cells contained <1% T cells as determined by N-SRBC rosetting and >50% B cells as judged by staining for surface membrane-associated IgM with a fluorescein-conjugated goat anti-human IgM antiserum. The pelleted cells from the first centrifugation were treated with isotonic NH_4Cl to lyse the N-SRBC and then passed over a nylon wool column. The population eluted from the column was highly enriched for T cells (90–95% N-SRBC rosetting).

Culture conditions for generation of immunoglobulin-secreting cells (ISC). Except where noted, cells were cultured in microtiter plates with U-wells (Dynatech Laboratories, Inc., Dynatech Corp., Alexandria, Va.). Routine cultures were carried out in triplicate with each microwell containing 1×10^5 PBM in 0.2 ml of culture medium. Mitogen (PWM, 10 μg/ml; SPA, 1 μg/ml) or an equivalent volume of HBSS as control was added to the wells, and they were incubated for 6–7 d at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. At the end of the incubation, cells from triplicate wells were pooled, washed, and resuspended in HBSS for assay.

Cultures of separated B- and T-cell populations were similarly carried out with each microwell containing 2.5×10^4 B cells alone or supplemented with 1×10^5 T cells. When the responsiveness of NAC was examined, cultures were carried out in triplicate in microtiter plates with flat-bottomed wells (Microtest II 3040, Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, Calif.) as previously described (36). In each well, 5×10^4 NAC were cultured with or without supplementation with 5×10^4 mitomycin-C-treated adherent cells.

Detection of ISC. ISC were detected with a previously described reverse hemolytic plaque assay (36) that made use of SPA-coated sheep erythrocytes. The lymphocytes to be tested and SPA-coated sheep erythrocytes were suspended in agarose (Indubiose A37, L-Industrie Biologique Francaise S. A., Gennevilliers, France) on microscope slides. After an initial 1-h incubation at 37°C, the slides were supported on Jerne racks and floated in developing anti-immunoglobulin antiserum (rabbit anti-human immunoglobulin [IgA + IgM + IgG], Cappel Laboratories, Inc., Downingtown, Pa.). After an additional 1-h incubation at 37°C, the slides were floated in a 1:20 dilution of guinea pig serum (Pel-Freeze Biologicals Inc., Rogers, Ark.) that previously had been absorbed with sheep erythrocytes. After a final 1-h incubation at 37°C, the hemolytic plaques that developed around ISC were enumerated. All data are expressed as the number of ISC/10^6 responding cells initially cultured.

RESULTS

Effect of Pen and $CuSO_4$ preincubation on PWM responsiveness of human PBM. Stimulation of human

PBM with PWM results in the generation of large numbers of ISC. Preincubation of PBM with either Pen (100 μg/ml) or $CuSO_4$ (2 μg/ml) alone had no significant effect on their subsequent PWM responsiveness as shown in Table I. However, a similar 2-h preincubation with the mixture of Pen and $CuSO_4$ significantly inhibited the capacity of PBM to generate ISC in response to PWM. In nine additional experiments, a similar 2-h preincubation with Pen and $CuSO_4$ was found to inhibit the PWM responsiveness of PBM by a mean of 93.8±3.2% (mean±SEM), whereas preincubation with either Pen (100 μg/ml) or $CuSO_4$ (2 μg/ml) alone had no significant inhibitory effect. Varying the PWM concentration from 0.1 to 100 μg/ml or varying the length of culture with PWM from 3 to 9 d failed to significantly increase the responsiveness of Pen- and $CuSO_4$-preincubated PBM. In addition, lack of responsiveness did not appear to result from cell death, as the viability of PBM judged by staining with ethidium bromide and fluorescein diacetate was comparable after preincubation with medium or with Pen and $CuSO_4$.

Pen and $CuSO_4$ preincubation not only inhibited the PWM responsiveness of human PBM but also inhibited their capacity to generate ISC in response to another T-cell-dependent polyclonal B-cell activator, SPA.[2] As shown in Table II, SPA responsiveness of human PBM was markedly depressed as a result of the preincubation with Pen and $CuSO_4$. In four of these experiments, PBM were also preincubated with either Pen (100 μg/ml) or $CuSO_4$ (2 μg/ml) alone and no significant inhibitory effect on subsequent SPA responsiveness was noted (data not shown).

To determine the minimum concentrations of Pen and $CuSO_4$ necessary to inhibit the capacity of PBM to generate ISC in response to polyclonal activators, experiments were carried out in which the amounts of Pen and $CuSO_4$ present during the preincubation were varied. As shown in Table III, preincubation of PBM with any of the concentrations of Pen or $CuSO_4$ alone had no significant effect on subsequent PWM responsiveness. When Pen and $CuSO_4$ were both present during the preincubation, inhibition of responsiveness was observed. The degree of inhibition appeared to be related both to the concentration of Pen and that of $CuSO_4$ present during the preincubation. Inhibition of responsiveness was observed after preincubations with $CuSO_4$ and concentrations of Pen (12.5 and 25 μg/ml) equivalent to those found in treated patients (39, 40). The largest concentration of $CuSO_4$ used in these experiments (2 μg/ml or 8 μM) is equivalent to about half the concentration found in normal human serum and less than one third of that found in the serum of patients with active rheumatoid arthritis (41, 42).

The cellular basis for the depressed responsiveness of Pen- and $CuSO_4$-preincubated PBM. Experiments were carried out to examine the cellular basis for the decreased capacity of Pen- and $CuSO_4$-preincubated PBM to generate ISC in response to mitogenic stimula-

[2] Lipsky, P. E. Staphylococcal protein A, a T-cell-regulated polyclonal activator of human B cells. Manuscript submitted for publication.

TABLE I

Effect of Preincubation on PWM Responsiveness of Human PBM

Preincubation* (2 h, 37°C)	PWM-induced ISC			
	Experiment 1	Experiment 2	Experiment 3	Experiment 4
	ISC/10⁶ cells			
Medium	18,375	8,400	3,750	9,000
Pen	16,950	8,665	3,550	11,175
$CuSO_4$	18,075	9,225	3,770	13,275
Pen + $CuSO_4$	75	300	50	2,775

* Pen, 100 μg/ml; $CuSO_4$, 2 μg/ml.

TABLE II

Effect of Pen and $CuSO_4$ Preincubation on the Capacity of PBM to Generate ISC

Preincubation (2 h, 37°C)	Mitogen-induced ISC	
	PWM	SPA
	*ISC/10⁶ cells**	
Medium	12,466±1,521	6,384±1,007
Pen + $CuSO_4$	1,735±686	90±43

* Mean±SEM of 16 separate experiments.

TABLE III

Effect of Pen and $CuSO_4$ Preincubation on the PWM Responsiveness of Human PBM

	Pen concentration*	PWM-induced ISC $CuSO_4$ concentration* (μg/ml)			
		0	0.5	1.0	2.0
	μg/ml	*ISC/10⁶ cells (× 10⁻³)*			
Experiment 1	0	8.4	8.4	8.9	9.2
	25	10.2	6.1	1.9	3.4
	50	8.7	4.6	1.5	0.1
	100	8.7	3.3	1.4	0.3
Experiment 2	0	20.5	22.8	21.8	20.3
	12.5	29.4	8.6	2.8	2.3
	25	21.7	0.1	0	0
	50	22.5	0.1	0	0

* PBM were preincubated for 2 h at 37°C in medium containing the concentrations of Pen and $CuSO_4$ indicated. After the preincubation, the cells were washed three times, suspended in fresh culture medium, and incubated for 6 d with PWM.

tion. Initially, the possibility that Pen and $CuSO_4$ preincubation augmented suppressor cell activity was examined. PBM were preincubated for 2 h at 37°C with medium alone or with Pen and $CuSO_4$, washed, and resuspended in fresh culture medium. As shown in Table IV, Pen- and $CuSO_4$-preincubated PBM exhibited markedly depressed responsiveness to both PWM and SPA compared with control PBM that had been preincubated in medium alone. When the two populations were co-cultured, Pen- and $CuSO_4$-preincubated PBM did not suppress the responsiveness of control PBM. On the contrary, the number of ISC generated in the co-cultures was greater than that predicted from the response observed when each population was cultured alone. These data indicate that the depressed responsiveness resulting from preincubation with Pen and $CuSO_4$ could not be explained by the induction of a suppressor cell or by nonspecific carry-over of the inhibitors into the culture.

The capacity of human PBM to generate ISC in response to PWM is dependent on the accessory cell function of Mϕ (36). Therefore, it was possible that the depressed responsiveness observed after preincubation with Pen and $CuSO_4$ resulted from an alteration of Mϕ function. To examine this possibility, PBM were cultured on glass petri dishes to separate them into Mϕ-depleted NAC and Mϕ-enriched adherent cells. Each population was then individually preincubated with the mixture of Pen and $CuSO_4$, washed, and co-cultured with PWM. A typical experiment is shown in Fig. 1. When PBM were depleted of Mϕ by glass adherence, their capacity to generate ISC in response to PWM was substantially reduced (35,600 → 12,800 ISC/10^6 cells). The addition of either control Mϕ that had been preincubated with medium alone or Pen- and $CuSO_4$-pre-

TABLE IV
Effect of Pen and $CuSO_4$ Preincubation on the Capacity of PBM to Generate ISC

Stimulus	Number of Experiments	Cells cultured*		
		PBM_{cont}	PBM_{cont} + $PBM_{Pen\ cu}$	$PBM_{Pen\ cu}$
		PWM-induced ISC/10^6 cells		
PWM	12	9,175±1,371	7,152±1,660	431±238
SPA	10	7,566±1,482	5,138±1,219	58±19

* PBM were preincubated for 2 h at 37° in medium alone (PBM_{cont}) or in medium containing 100 μg/ml Pen and 2 μg/ml $CuSO_4$ ($PBM_{Pen/cu}$). After the preincubation, the cells were washed three times and suspended in fresh culture medium. The cells were then aliquoted into the wells of microtiter plates and incubated either alone (1×10^5/microwell) or mixed together (0.5×10^5 PBM_{cont} + 0.5×10^5 $PBM_{Pen/cu}$) with mitogen for 6 d before assay. Data represent the mean±SEM of the number of experiments indicated.

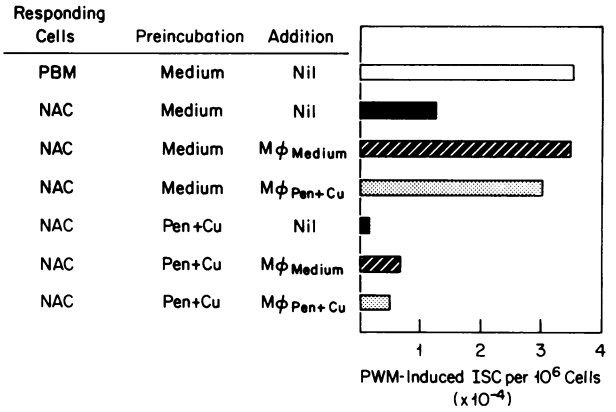

FIGURE 1 Lack of effect of Pen and $CuSO_4$ preincubation on the accessory cell function of Mϕ. PBM were separated into NAC and Mϕ by adherence to glass petri dishes. Each was then preincubated for 2 h at 37°C with medium alone as control or with the mixture of Pen (100 μg/ml) and $CuSO_4$ (2 μg/ml). After washing, the cells were aliquoted into flat-bottomed microtiter wells (5×10^4 NAC with or without 5×10^4 Mϕ), incubated with PWM for 6 d, and assayed for the number of ISC.

incubated Mϕ restored PWM responsiveness to the NAC. In contrast, when the NAC were preincubated with the mixture of Pen and $CuSO_4$, their capacity to generate ISC in response to PWM was further reduced and could not be rescued by either Mϕ population. These data indicate that the preincubation with Pen and $CuSO_4$ diminished the PWM responsiveness of cells in the nonadherent population, but had little inhibitory effect on the functional capability of the Mϕ.

The differentiation of ISC from B-cell precursors in response to PWM and SPA is dependent on the activity of a subpopulation of helper T cells (43).[2] Experiments were, therefore, carried out to determine whether the mixture of Pen and $CuSO_4$ might alter helper T-cell activity. PBM were preincubated with Pen and $CuSO_4$ and, as shown in Fig. 2, the response to both SPA (experiment 1) and PWM (experiments 2 and 3) was markedly decreased. When the preincubated PBM were then co-cultured with a purified population of fresh autologous T cells, their capacity to generate ISC in response to mitogenic stimulation was restored. These experiments suggested that Pen and $CuSO_4$ preincubation had altered helper T-cell activity but had not depressed B-cell function.

To examine this possibility more fully, populations enriched for B and T cells were prepared. As shown in Table V, B cells, when cultured alone, were unable to differentiate into ISC in response to PWM. When these B-cell cultures were supplemented with control T cells, large numbers of ISC were generated. Markedly decreased numbers of ISC were found when B cells were co-cultured with Pen- and $CuSO_4$-preincubated T cells, indicating that preincubation of T cells with Pen and

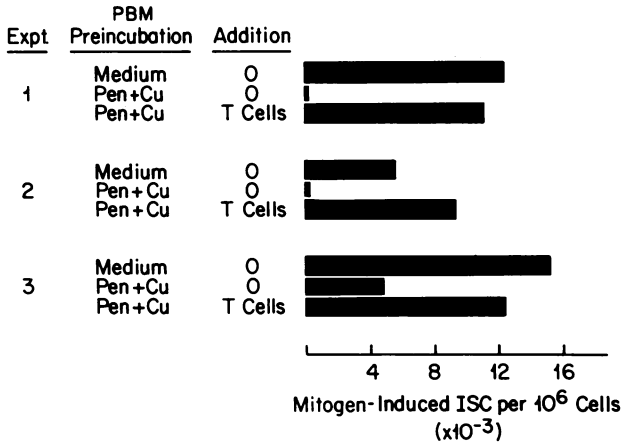

FIGURE 2 T-cell rescue of the depressed responsiveness of Pen- and $CuSO_4$- preincubated PBM. PBM were preincubated for 2 h at 37°C with medium or with the mixture of Pen (100 μg/ml) and $CuSO_4$ (2 μg/ml). After the preincubation, the cells were cultured either alone (1×10^5 cells/well) or co-cultured with fresh autologous T cells (5×10^4 PBM with 5×10^4 T cells). After a 6-d incubation with SPA (experiment 1) or PWM (experiments 2 and 3), the number of ISC was determined.

$CuSO_4$ had profoundly decreased their capacity to function as helper cells. The diminished helper activity of these T cells could not be ascribed to nonspecific carry-over of the inhibitors or to an augmentation of suppressor cell function because Pen- and $CuSO_4$-preincubated T cells did not diminish responses supported by control T cells (Table V).

Finally, experiments were carried out to determine whether the inhibitory action of Pen and $CuSO_4$ was specific for helper T cells or whether the responsiveness of B cells was also altered. Populations enriched for B and T cells were prepared, individually preincubated with medium as control or Pen and $CuSO_4$, and co-cultured with PWM to determine the effect of the preincubation on the function of each cell type. In each of the experiments shown in Fig. 3, control B cells failed to respond to PWM, although supplementation with control T cells resulted in the generation of large numbers of ISC. As previously shown, preincubation of T cells with Pen and $CuSO_4$ markedly depressed their capacity to function as helper cells. In contrast, preincubation of B cells with Pen and $CuSO_4$ had little effect on their functional activity. Thus, in each experiment, few ISC were generated without T-cell supplementation, whereas co-culture with control T cells led to the generation of similar numbers of ISC to those found in cultures containing control B cells. As before, Pen- and $CuSO_4$-preincubated T cells were ineffective in providing help. It should be noted that in these experiments the cells were incubated with Pen at a concentration of 12.5 (experiments 2 and 3) or 25 μg/ml (experiment 1 and 4) in the presence of $CuSO_4$ (2 μg/ml). Preincubations with higher concentrations of Pen (50 or 100 μg/ml) in the presence of $CuSO_4$ did interfere with B- as well as T-cell function. However, at concentrations of Pen (<25 μg/ml) approximating that found in the serum of treated patients (39, 40), Pen and $CuSO_4$

TABLE V
Effect of Pen and $CuSO_4$ Preincubation on Helper T-Cell Function

Addition to B-cell cultures*	PWM-induced ISC		
	Experiment 1	Experiment 2	Experiment 3
	ISC/10⁶ B cells		
Nil	200	1,400	400
Control T cells	14,400	120,000	86,800
Pen/Cu T cells	400	6,000	1,600
Control + Pen/Cu T cells	18,000	112,800	82,000

* T cells were preincubated for 60 min at 37°C with a mixture of Pen (100 μg/ml) and $CuSO_4$ (2 μg/ml) or with medium alone as control. B cells (2.5×10^4/microwell) were mixed with each T-cell population (1×10^5/microwell) as noted, cultured with PWM for 6 d, and the number of ISC determined.

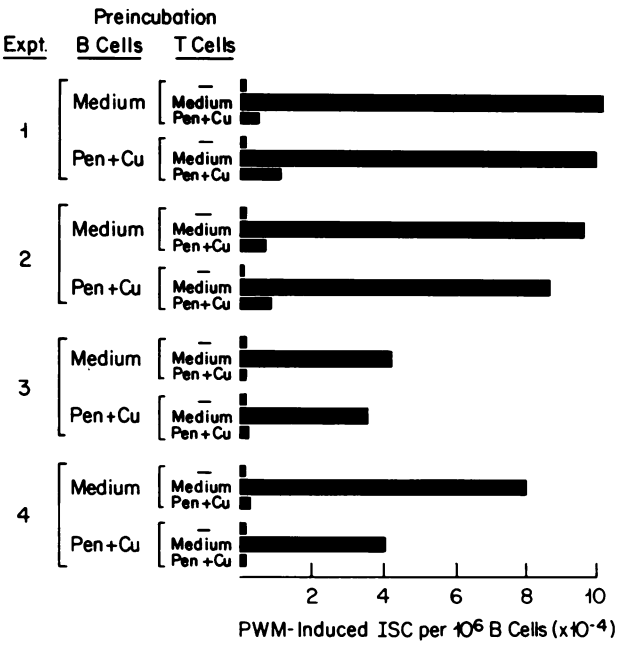

FIGURE 3 Pen and $CuSO_4$ preincubation inhibits helper T-cell but not B-cell function. Populations enriched for B or T cells were preincubated for 1 h at 37°C with the mixture of Pen (12.5 μg/ml in experiments 2 and 3 or 25 μg/ml in experiments 1 and 4) and $CuSO_4$ (2 μg/ml), washed, and co-cultured as indicated. Cultures contained 2.5×10^4 B cells with no additional T cells, with 1×10^5 control T cells, or with 1×10^5 Pen plus $CuSO_4$-preincubated T cells. After a 6-d incubation with PWM, the number of ISC was determined.

inhibited helper T-cell function without altering B-cell responsiveness.

DISCUSSION

A number of immunological processes play an important role in the pathogenesis of rheumatoid arthritis (44). One such mechanism involves the production of rheumatoid factors that interact with IgG to form immune complexes, activate complement, and trigger a number of the pathogenic processes characteristic of rheumatoid inflammation. One of the hallmarks of therapy with Pen is the capacity of this drug to decrease rheumatoid factor titers (1, 9, 24, 25). It is unlikely that this results from a direct action of Pen on IgM rheumatoid factors because the serum concentration of Pen attained in treated patients (39, 40) is many-fold less than that needed to dissociate macroglobulins (6). Rather, it appears more likely that the action of Pen results from its capacity to alter the function of the cells involved in antibody production.

We have previously shown that Pen inhibits the capacity of human peripheral blood T cells to proliferate in response to nonspecific phytomitogens (32). Inhibition required that the cells be exposed to Pen in the presence of copper ions; a number of other metal salts were ineffective in potentiating the inhibitory effect. In the experiments presented in this report, Pen was also found to inhibit the capacity of PBM to generate ISC in response to in vitro stimulation with T-cell-dependent polyclonal B-cell activators. Again, inhibition was only observed when cells were incubated with Pen in the presence of copper ions. Moreover, the inhibition of PBM responsiveness resulting from the in vitro preincubation with the Pen and $CuSO_4$ mixture persisted after the cells were washed free of the inhibitors. As a result, the various populations of cells involved in the generation of ISC could be individually exposed to the inhibitors to determine the cellular locus of action of Pen. In addition, the irreversible nature of the inhibition made it unnecessary for Pen to be continuously present during the entire culture period. This ruled out the possibility that the inhibitory action of Pen resulted from its capacity to bind sulfhydryl compounds in the medium and, thus, make them unavailable to the cells as has been suggested to occur in other systems (45).

Previous studies from this laboratory indicated that Pen in the presence of $CuSO_4$ inhibited the capacity of T lymphocytes to proliferate in response to nonspecific phytomitogens (32). Specificity for the inhibition was suggested by the finding that the capacity of Mϕ to function as accessory cells necessary for the induction of T-cell proliferation was not diminished. In the studies reported here, the ability of Mϕ to serve as requisite accessory cells for the generation of ISC in vitro (36) was also not altered by Pen. The general conclusion that Pen does not inhibit Mϕ function is supported by Binderup et al. (46) who found that Pen alone did not inhibit rat macrophage function. On the contrary, some of the activities of these cells were actually enhanced. The conclusion that Pen selectively inhibits T-cell function is further supported by the current observation that the Pen and $CuSO_4$ preincubation did not alter the capacity of B cells to undergo terminal differentiation into ISC after appropriate stimulation.

Inhibition of helper T-cell function was observed with concentrations of both Pen and copper that were similar to those likely to be found in serum of treated patients. Thus, the maximum concentration of copper employed (8 μM) was only half that found in normal serum and much less than that found in patients with rheumatoid arthritis who tend to have markedly elevated concentrations of both serum and synovial fluid copper (41, 42, 47, 48). However, the vast majority of serum and synovial fluid copper does not occur in a free exchangeable form but, rather, as an integral part of the cuproprotein ceruloplasmin (41, 42, 47–49). Thus, the relationship of the effective concentrations of copper used in these in vitro studies to copper levels found in vivo is less clear. A central issue, therefore, involves the capacity of Pen to interact with ceruloplasmin copper in mediating the inhibition of T-cell function. In preliminary studies, we have found that purified human ceruloplasmin itself is not inhibitory but, at physiological concentrations, can markedly augment the capacity of Pen to inhibit T-cell function. This finding provides additional evidence for the in vitro relevance of the current in vitro observations.

Inhibition of PBM responsiveness was seen routinely after preincubation with 12.5 μg/ml of Pen in the presence of $CuSO_4$. In some experiments using purified cell populations, inhibition of helper T-cell activity could be observed after preincubations with as little as 6 μg/ml of Pen. Recent studies have shown that these concentrations are within the range of serum concentrations found in treated patients (39, 40), which may reach as much as 20 μg/ml after a single oral dose of Pen. Therefore, the inhibition of helper T-cell activity observed in the current in vitro studies was induced by preincubation of cells with concentrations of both Pen and $CuSO_4$ that might well be expected to be found in vivo in patients treated with Pen.

A number of observations in treated patients support the idea that Pen might exert an immunosuppressive action in vivo. Thus, therapy with Pen has been shown to decrease antiglobulin titers (1, 9, 24, 25), levels of circulating immune complexes (26–28), and concentrations of serum immunoglobulins (27–30). However, attempts to confirm the immunosuppressive action of Pen in experimental animals has led to conflicting re-

sults. A number of studies have shown that Pen might inhibit (50–52), enhance (53), or have no effect (18, 54) on the immune response of intact animals. More recently, Hunneybull et al. (31) have shown that administration of Pen can have a significant immunosuppressive effect in vivo. They showed that rabbits treated orally with 15 mg/kg of Pen, a dose equivalent to that used in patients with rheumatoid arthritis, exhibited a depressed in vivo antibody response to immunization with egg albumin. This was especially marked late in the immune response and seemed preferentially to effect high-avidity IgG antibody production. Concomitant with this, there was a more striking decline in cell-mediated immunity (55). These data suggested that prolonged administration of Pen had depressed T-cell function in vivo, resulting in inhibition of both cell-mediated immunity and the high-avidity IgG component of the humoral immune response. Our own results support the idea that the major action of Pen is to inhibit T-cell function. Previous work has shown that Pen inhibits the capacity of T cells to proliferate in vitro in response to mitogenic stimulation (32), whereas the current studies establish that Pen can selectively inhibit helper T-cell function without altering B-cell responsiveness or the accessory cell function of Mϕ. It is possible that Pen may also interfere with the activity of other functional subpopulations of T cells. For example, the administration of Pen has been shown to be associated with the development of a number of side effects, such as myasthenia gravis (52, 56) and pemphigus foliaceus and vulgaris (57), that involve the development of autoantibodies. It is possible that in such patients suppressor rather than helper T cells are uniquely sensitive to the inhibitory action of Pen. Studies using cells from treated patients will be needed to evaluate this possibility.

ACKNOWLEDGMENTS

We wish to thank Ms. Patricia Thompson for her expert technical assistance and Ms. Brenda Guest and Ms. Bonita Walker for preparing the manuscript.

This work was supported by U. S. Public Health Service Program Project grant AM-09989 and an Arthritis Foundation Clinical Center grant.

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