Inhibition of R5X4 Dualtropic HIV-1 Primary Isolates by Single Chemokine Co-receptor Ligands

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The susceptibility of HIV-1 to chemokine-mediated inhibition may be lost as a consequence of the expanded usage of chemokine co-receptors frequently occurring in clade B isolates obtained from individuals with advanced disease. Since chemokine-based immune intervention is under intense investigation, it is crucial to determine its potential effect on primary dualtropic HIV isolates characterized by simultaneous utilization of CCR5 and CXCR4 chemokine co-receptors (R5X4 viruses).

In the present study, the CCR5 binding chemokine regulated upon activation normal T cell expressed and secreted (RANTES) strongly inhibited the replication of two of eight primary R5X4 viruses in mitogen-activated primary peripheral blood mononuclear cells (PBMC). The CXCR4 antagonist AMD3100 efficiently suppressed the replication of other two HIV isolates, whereas the remaining four viruses were partially inhibited by treatment with either RANTES or AMD3100. The potency of chemokine-mediated inhibition was influenced by PBMC donor variability, but it was usually independent from the levels of expression of CCR5 or CXCR4. Dual co-receptor usage was maintained by the viruses after two serial passages on U87.CD4 astrocytic cell lines expressing exclusively either CCR5 or CXCR4. The gp120 env variable domains were sequenced before and after passages on U87.CD4 cells. Virus replication into U87.CD4-CXCR4 cells did not result in changes in the V3 region but perturbed the dominant env V4 sequence. Interestingly, double passage onto U87.CD4-CXCR4 cells determined the loss of susceptibility to RANTES inhibition. In conclusion, interference with CCR5 may efficiently inhibit the replication of at least some dualtropic HIV-1 strains, whereas forced CXCR4 usage may result in viral escape from CCR5-dependent inhibitory effects.© 2001 Academic Press

INTRODUCTION

CC-chemokines, and particularly regulated upon activation normal T cell expressed and secreted (RANTES), protect cells from infection with macrophage-tropic non-syncytium inducing (NSI) HIV-1 strains using CCR5 (R5 viruses) as entry co-receptor (Alkhatib et al., 1996; Berger et al., 1998; Choe et al., 1996; Cocchi et al., 1995; Deng et al., 1996). Conversely, the CXC-chemokine stromal-cell-derived factor-1 (SDF-1α), the unique ligand of CXCR4 (Bleul et al., 1996), inhibits entry and replication of T-tropic SI (X4) viruses, but not of R5 HIV-1 strains (Feng et al., 1996; Oberlin et al., 1996). However, RANTES has been also reported to enhance replication of X4 monotropic and dualtropic R5X4 viruses (Dolei et al., 1998; Gordon et al., 1999; Kinter et al., 1998; Trkola et al., 1999). These findings suggested the possibility that RANTES and other CCR5 binding chemokines could force HIV-1 to evolve into more virulent forms in vivo by inducing a phenotypic switch from CCR5 to CXCR4 usage as a result of both inhibition of R5 virus entry and enhancement of X4 HIV replication (Michael and Moore, 1999; Mosier et al., 1999). In support of this hypothesis, loss of sensitivity to RANTES-mediated inhibitory effects on virus replication has been documented in sequential isolates obtained from infected individuals, extending their co-receptor usage from CCR5 to CXCR4 along with disease progression (Bjorndal et al., 1997; Scarlatti et al., 1997).

Aim of our study was to investigate the susceptibility to chemokine-mediated inhibitory effects of R5X4 dualtropic primary isolates with respect to treatment with either RANTES or AMD3100, a selective CXCR4 antagonist (Schols et al., 1997). Different donors’ PBMC, levels of chemokine co-receptor expression, and variations in their gp120 env sequences before and after forced replication in either CCR5- or CXCR4-expressing cells were studied.

RESULTS

RANTES and AMD3100 inhibit the replication of primary R5X4 dualtropic isolates

Eight viruses were selected by both CCR5 and CXCR4 usage (R5X4) according to their fusogenic and replicative capacities in U87.CD4 cell lines. One hundred microliters...
of undiluted primary HIV-1 isolates was adsorbed to PHA blasts (10^6 cells/ml) of different HIV seronegative individuals. RANTES or AMD3100 was then added on independent wells and supplemented a second time 72 h after infection. RANTES (100 ng/ml) suppressed the replication of two (SR-2 and SR-9) of eight R5X4 HIV strains (Fig. 1). No increased levels of suppression were observed with 1000 ng/ml of the chemokine, whereas its inhibitory effect was lost between 10 and 1 ng/ml with all the tested viruses (data not shown). Therefore, the range of RANTES concentration that inhibited the R5X4 replication in PHA blasts was similar to that required for inhibition of R5 monotropic viruses (Cocchi et al., 1995; Dragic et al., 1996; Jansson et al., 1996). SR-4 and SR-6 R5X4 primary isolates were insensitive to RANTES inhibitory effects, whereas the CXCR4 antagonist AMD3100 (1 μg/ml) suppressed their replication in PHA blasts. Four additional primary isolates (SR-3, SR-5, SR-7, and SR-8) were partially inhibited by treatment with either RANTES or AMD3100 (Fig. 1).

To demonstrate whether RANTES inhibitory effect on dualtropic HIVs was dependent upon inhibition of viral entry, the region spanning from R to U5 within the 5' LTR (R-U5) was amplified. RANTES prevented proviral DNA synthesis of both R5 and R5X4 HIVs, at 24 and 48 h p.i. consistently with interference of viral entry (data not shown).

![Fig. 1](image1.png)

**FIG. 1.** Patterns of interference with primary dualtropic virus replication by RANTES and AMD3100. RANTES efficiently inhibited the replication of SR-2 and SR-9, whereas AMD3100 suppressed SR-4 and SR-6 virus spreading in human PHA blasts (top). The remainder viruses showed intermediate susceptibility to either agent (bottom). Results are expressed as means ± SE of RT activities of culture quadruplicates and triplicates for untreated cultures and for treated cultures, respectively, 7 days p.i. in a single experiment.

Donor variability and cell-surface expression of HIV co-receptors as determinants of R5X4 virus susceptibility to RANTES or AMD3100

To characterize the broad range of inhibitory effects observed with RANTES and AMD3100, three primary isolates (SR-2, SR-3, and SR-4) were selected as prototypes of the observed patterns. The potency of RANTES inhibitory effects on R5X4 virus replication varied substantially among PHA blasts of independent donors, ranging from complete suppression in the case of SR-2 (median inhibition: 72%) to poor inhibition for SR-3 and SR-4 (median inhibitions: 32.5 and 19%, respectively) (Fig. 2). Conversely, AMD3100 (1 μg/ml), tested in parallel cultures, efficiently inhibited SR-4 replication (median

![Fig. 2](image2.png)

**FIG. 2.** Influence of PBMC donor-to-donor variability on RANTES and AMD3100 inhibitory effects on dualtropic HIV isolates. The percentage inhibition of peak of RT activities (occurring between 7 and 12 days p.i.) by either RANTES (left) or AMD3100 (right) over untreated cultures is shown. The whiskers indicate the range of the data with the boxes extending from the 25th to the 75th percentile, whereas the horizontal lines are the median values. Statistical analysis was carried out according to the Kruskal–Wallis computed with the Dunn’s multiple comparison test.
inhibition of 96.5%), whereas SR-2 and SR-3 replication was partially suppressed by this agent (median inhibitions: 61 and 65.5%, respectively, Fig. 2).

To verify whether RANTES and AMD3100 suppression of dualtropic virus replication was correlated to preferential expression of CCR5 or CXCR4 co-receptors, flow cytometric analyses of PBMC were carried out in freshly isolated PBMC and PHA blasts from seven additional donors. Mitogenic stimulation induced up-regulation of both CCR5 and CXCR4 on CD4 \(^+\) T cells with median fold induction compared to unstimulated cells of 6- and 10-fold, respectively (not shown). As shown in Fig. 3, a stronger inhibition of SR-2 and SR-3 virus replication by RANTES was observed when the donors’ PHA blasts expressed the highest levels of CCR5 and the lowest amounts of CXCR4, although this correlation was not statistically significant. Conversely, AMD3100 suppressed almost completely the replication of SR-4 in seven of seven PBMC donors (median suppression: 94%), whereas it blocked SR-2 and SR-3 replication only in part (with median inhibitions of 54 and 77%, respectively) in a donor-dependent fashion (Fig. 3). Interestingly, a negative correlation was found between the potency of inhibition of SR-4 replication by AMD3100 and the levels of CCR5 expression (Spearman’s \( r = -0.89, P = 0.01 \)). Therefore, the levels of chemokine receptor expression on the cell surface may influence in part the susceptibility to different primary isolates to co-receptor inhibitors.

**Co-receptor usage and characterization of gp120 Env variable regions before and after passage onto U87.CD4 cells**

To test whether the relative susceptibility of SR-2, SR-3, and SR-4 primary isolates to RANTES or AMD3100 inhibition was dependent upon the dominance of monotropic R5 or X4 viral variants present in their swarms, these viruses were passaged twice on U87.CD4 cell lines expressing either CCR5 or CXCR4 co-receptors. The viruses resulting from the second passage on these cell lines were analyzed for co-receptor usage. Of interest, all the three second-passage viruses maintained dual co-receptor usage regardless of which co-receptor was utilized for spreading in U87.CD4 cells (Fig. 4).

The gp120 Env variable regions play a major role in the interaction with both CD4 and chemokine receptor (Riz-
The most heterogeneous as compared to other viral isolates, whereas a predominant species was present in 7 of 10 clones of SR-2 with 3 clones containing a few variations as compared to the predominant species. SR-4 was the most homogenous virus, in that only one minor variant was detected (Fig. 5). Of interest, the percent similarity to ADA of the env V3 predominant species of the dual-tropic viruses ranged from 74 (SR-4) to 87% (SR-3), whereas the percent homology with the X4 prototype NL4-3 V3 env region showed values from 50 (SR-2) to 58% (SR-3; data not shown). Thus, our dual-tropic HIV isolates appeared more related to a macrophage-tropic rather than to a TCLA virus in their env V3 sequence. The sum of positive and negative charges of env V3 was also evaluated. In this regard, charges ranging from +2 to +5 are typical of macrophage-tropic strains, whereas values greater than +7 characterize T-tropic viruses (Ugolini et al., 1999). The overall range of the net charges of env V3 of our primary isolates was from +4 to +8, whereas none of the single variants reached the values characteristic of X4 viruses such as NL4-3 and MN. Single aa positions have been associated to viral tropism. For example, a change at position 11 from a neutral to a positively charged aa has been associated to both the NSI to SI phenotypic switch (De Jong et al., 1992; Fouchier et al., 1992) and to the extension of coreceptor usage from CCR5 to CXCR4 (Connor et al., 1997; Scarlatti et al., 1997). As expected, SR-2 and SR-4 isolates had an arginine (R) in the predominant variant in this position (Fig. 5). However, the less-frequent viral variants cloned from the primary isolates were characterized by neutral aa such as serine (S) or glycine (G). Unexpectedly, SR-3 showed a neutral S at position 11 in all clones despite its SI phenotype and R5X4 usage; its V3 net charge, however, was estimated +8, and therefore it can be considered more similar to X4 than R5 viruses (Fig. 5). Moreover, isoleucine (I) at position 30, conferring CCR5 usage, was found in 100% of clones obtained from dual-tropic viruses, as previously shown (Chan et al., 1999; Hung et al., 1999). After two serial passages on U87.CD4 cells expressing either CCR5 or CXCR4, the I residue at position 30 was maintained. The I at position 27, also involved in the CCR5 usage (Hung et al., 1999), was present in SR-3 isolate, whereas it was present only in 2 of 10 clones of SR-2, but it was selected after passage on U87.CD4 expressing CCR5 (Fig. 5). A selection of minor quasispecies present in the original isolate occurred for SR2 and SR3 after R5/R5 passages, whereas emergence of a mutant in V3 was noted for SR4.

The aa alignments of the V4/V5 variable regions of gp120 Env are also shown in Fig. 5. The serial passages on U87.CD4 expressing CXCR4 did not change the sequence of the predominant species with the notable

![FIG. 4. Forced co-receptor usage and dualtropism. Serial passages of SR-2, SR-3, and SR-4 primary HIV-1 isolates on U87.CD4 expressing either CCR5 or CXCR4. The range of peak RT activities were scored as follows: +++++ = ≥5000 cpm/µl; +++ = 2000 – 5000 cpm/µl; ++ = 1000 – 2000 cpm/µl; + = ≤1000 cpm/µl.](image-url)
exception of the V4 region of SR-2 strain. In addition to several aa changes, insertion of 5 aa in addition to several other aa changes within V4 occurred in SR-2X4/X4 (Fig. 5).

Dissociation of co-receptor usage from RANTES inhibitory effect on R5X4 HIV-1 isolates

SR-2 and SR-3 primary isolates changed their V1/V2 sequences after passages on U87.CD4-CCR5 (R5/R5) or U87.CD4-CXCR4 cells (X4/X4). However, the replication of SR-2 and SR-3 virions after two serial passages onto U87.CD4-CCR5 cells was still inhibited by RANTES. In sharp contrast, both SR-2 and SR-3 viruses completely lost their sensitivity to RANTES inhibition after two serial passages on U87.CD4-CXCR4 (Fig. 6). These results indicate that the env V3 region is not the only determinant of RANTES inhibition of dualtropic viruses, whereas the sequence variation in V4 could be responsible of the loss of RANTES inhibitory effects on SR-2 replication. However, the Env structural requirement for entry of SR-3 might be different since no variations were detected in all SR-3 gp120 Env variable regions as compared to the original primary isolate.

FIG. 5. Amino acid (aa) sequence alignment of the variable regions of gp120 Env, including V1/V2, V3, and V4/V5 of SR-2, SR-3, and SR-4 viral isolates before and after two serial passages on U87.CD4-CCR5 (R5/R5) or U87.CD4-CXCR4 cells (X4/X4). The V3 sequences from 10 to 13 independent clones per each primary isolate are reported. The observed frequency of each sequence is reported at the left, whereas their net charges are indicated on the right. Dots denote aa identity, whereas dashes indicate gaps to optimize the alignments.

FIG. 6. Loss of susceptibility to RANTES-mediated inhibition in dualtropic viruses passaged twice on U87.CD4-CXCR4 cells. RANTES-mediated inhibition of SR-2 and SR-3 infection in PHA-blasts before (I° Isolate) and after two passages on either U87.CD4-CCR5 (R5/R5) or U87.CD4-CXCR4 (X4/X4). The percentage inhibition of peak of RT activities (12 days p.i.) by RANTES over untreated cultures is shown.

SR-2 and SR-3 primary isolates changed their V1/V2 sequences after passages on U87.CD4 expressing CCR5, and SR-2 changed its V4 after passage on U87.CD4-CXCR4 cells. However, the replication of SR-2 and SR-3 virions after two serial passages onto U87.CD4-CCR5 cells was still inhibited by RANTES. In sharp contrast, both SR-2 and SR-3 viruses completely lost their sensitivity to RANTES inhibition after two serial passages on U87.CD4-CXCR4 (Fig. 6). These results indicate that the env V3 region is not the only determinant of RANTES inhibition of dualtropic viruses, whereas the sequence variation in V4 could be responsible of the loss of RANTES inhibitory effects on SR-2 replication. However, the Env structural requirement for entry of SR-3 might be different since no variations were detected in all SR-3 gp120 Env variable regions as compared to the original primary isolate.

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**TABLE 1**

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**FIG. 6.** Loss of susceptibility to RANTES-mediated inhibition in dualtropic viruses passaged twice on U87.CD4-CXCR4 cells. RANTES-mediated inhibition of SR-2 and SR-3 infection in PHA-blasts before (I° Isolate) and after two passages on either U87.CD4-CCR5 (R5/R5) or U87.CD4-CXCR4 (X4/X4). The percentage inhibition of peak of RT activities (12 days p.i.) by RANTES over untreated cultures is shown.
DISCUSSION

In the present study, we investigated the susceptibility of a pool of randomly selected primary R5X4 dualtropic HIV-1 isolates to inhibition by chemokine receptor ligands of their replicative ability in PHA blasts of different seronegative donors. Four of eight viral isolates were strongly inhibited either by RANTES (2/4) or AMD3100 (2/4), binding to CCR5 and CXCR4, respectively, whereas the remaining four isolates showed partial susceptibility to either co-receptor inhibitors. Donor variability and levels of cell-surface expression of CCR5 and CXCR4 influenced the degree of inhibitory effects observed, however, without substantial changes of the observed patterns. Although sequence variability was observed in the gp120 Env V3 variable loops of three prototypic primary isolates, their double passage onto U87.CD4 cells expressing exclusively CCR5 or CXCR4 did not sort out monotropic viral variants in terms of co-receptor usage. However, forced use of CXCR4 resulted in the loss of susceptibility to RANTES inhibitory effects in previously sensitive HIV isolates. Molecular analysis suggests that V4 rather than V3 domain of gp120 Env may be an important determinant of sensitivity and escape from chemokine-mediated control of virus replication.

It is well documented that clade B HIV acquires multiple chemokine receptors usage during disease progression (Connor et al., 1997; Scarlatti et al., 1997). Extension to CXCR4 utilization in addition to CCR5 has been reported to coincide with the loss of chemokine-mediated inhibition of viral replication (Jansson et al., 1996). With the caveat that only a small number of primary isolates were investigated, our findings suggest that single agonists or antagonists of CCR5 or CXCR4 can efficiently inhibit dualtropic viruses. Amino-terminal modifications to RANTES have been shown to increase its potency as inhibitor of HIV replication (Mosier et al., 1999; Simmons et al., 1997). However, these compounds were not tested in the present study. No clear-cut correlation was observed between the levels of CCR5 or CXCR4 expression on the PHA blast cell surface and the potency of RANTES inhibition of SR-2 and SR-3. On the other hand, the potency of AMD3100 inhibition of SR-4 was significantly correlated to lower CCR5 expression, suggesting that CXCR4 could be preferentially used by SR-4. In this regard, co-receptor competition for association with CD4 has been recently shown to affect HIV entry and inhibition by chemokine receptor agonists and antagonists (Lee et al., 2000).

The dualtropic nature of the gp120 Env of the HIV-1 isolates was not resolved into R5 and X4 monotropic variants by sequential passage in cell lines expressing exclusively either CCR5 or CXCR4. However, two passages in CXCR4 expressing cell lines determined the loss of their susceptibility to the inhibitory effect of RANTES of both SR-2 and SR-3 viruses. In this regard, it has been suggested that CC-chemokines may enhance the replication of SI HIV-1 strains in CD4+ T lymphocytes (Dolei et al., 1998; Gordon et al., 1999; Kinter et al., 1998; Trkola et al., 1999). However, these enhancing effects of RANTES have been observed either at low m.o.i. (Kinter et al., 1998) or after incubation of CD4+ cells with high (1–10 μg/ml) concentrations of RANTES 48 h prior to infection (Gordon et al., 1999), conditions that were not investigated in our study. However, the observed loss of susceptibility to RANTES inhibitory effect after passage in U87.CD4.CXCR4 cells suggests that forced utilization of this chemokine receptor may profoundly alter the responsiveness to CCR5 binding chemokines as observed here with RANTES, at least in terms of inhibition of HIV infection.

Recently, RANTES has been shown to suppress the replication of the chimeric 89.6-SF162 R5X4 virus ex vivo in human lymphoid tissue (Glushakova et al., 1999), suggesting that some dualtropic viruses may be more similar to R5 in terms of viral spreading and chemokine sensitivity than others (Glushakova et al., 1999). In the present study, although primary isolates containing several quasispecies were used, dualtropic SR-2 and SR-3 HIV isolates were more related to NSI rather than to SI viruses in regard of homology to the gp120 env V3 sequence of the ADA and NL4-3 prototype HIV. Also the net charge, and critical aa positions such as I at position 27 and 30 were more similar to NSI than SI laboratory-adapted strains (Chan et al., 1999; Hung et al., 1999). Conversely, our SR-4 primary HIV was poorly inhibited by RANTES but efficiently blocked by AMD3100 particularly in those PHA blasts that expressed the lowest CCR5 levels on their cell surface. Sequencing of the gp120 env V3 before and after passages did not result in significant changes within the V3 region. In addition, V1/V2 region(s) confirmed that changes did occur during in vitro passages of SR-2 and SR-3 on U87.CD4 expressing CCR5. In contrast, the gp120 Env V4 region of SR-2 changed substantially after two serial passages on U87.CD4 cells expressing CXCR4, whereas infection on U87.CD4 expressing CCR5 cells did not alter the predominant species of the primary viral isolate. These observations indicate that sequences outside V3 through V4 can disable RANTES inhibitory effects, supporting previous observations indicating that co-receptor utilization and tropism of dualtropic viruses are determined by complex interactions among multiple Env domains (Cho et al., 1998; Smyth et al., 1998).

The mechanism underlying the profound inhibition of some dualtropic HIVs by single chemokines or chemokine antagonists, a phenomenon already noted and discussed by others (He et al., 1997), remains speculative. Heterologous interactions among different chemokine receptors such as CCR5, CXCR4, and CCR2b (at least in its allelic variant V64I) leading to inhibition of co-receptor-dependent HIV entry have been recently reported.
(Mellado et al., 1999). Therefore, CCR5 occupancy by RANTES may not only prevent usage of this co-receptor by the virus, but also influence in trans the ability of Env to interact with CXCR4. The observation that a CXCR4 inhibitor such as AMD3100, devoid of signaling capacity, exerted similar effects on our viruses than those observed with RANTES does not support this interpretation. On the other hand, the recent observation that pertussis toxin and its B-oligomer subunit exert direct inhibitory effects on R5 HIV entry and replication (Alfano et al., 1999, 2000) challenges the "dogma" that chemokine-mediated inhibition of HIV infection does not require signal transduction.

Finally, the demonstration that infection and replication of at least some dualtropic viruses are inhibited by RANTES or AMD3100 fully supports the concept that inhibitors of chemokine receptors are a promising new class of antiviral agents potentially capable of preventing infection in highly exposed individuals or limiting HIV spreading in infected individuals.

MATERIALS AND METHODS

Viruses and cells

Primary HIV isolates from individuals with progressing HIV-1 disease were generated by co-cultivation of 4 × 10^6 peripheral blood mononuclear cells (PBMC) with a mixture of 3 × 10^6 allogeneic phytohemagglutinin (PHA-P, Sigma Chemical Corp., St. Louis, MO)-stimulated PBMC (PHA blasts) from two uninfected individuals in 10 ml of complete RPMI 1640 medium (Bio Whittaker, Verviers, Belgium) containing 10% FCS (Sigma) and 20 U/ml PHA blasts were stained with PE-conjugated anti-CD4 mAb, or FITC-conjugated anti-CXCR4 (clone 12G5, R&D Systems) mAbs, or FITC/PE-conjugated isotype-matched control mAbs. Stained cells were then analyzed by flow cytometry (FACScan, Becton Dickinson).

PCR and sequencing

Viral RNA was extracted from 40 μl of supernatant by the guanidinium thiocyanate method (Menzio et al., 1998) and retrotranscribed by poly d(N),. The following primers were used for amplification of the V1-V2 regions: sense (5′-CATATACAAGTTATGGGAC-3′) and antisense (5′-GCACAATAATGTAGGAAATTGG-3′). For the V3 region, the following primers were used: sense V31 (5′-TCAGCACAGTACAATGTACACATGGAAT-3′) and antisense V32 (5′-AGTAGAAAAATTCCTCCACACATTAAA-3′) (Menzio et al., 1998). Two microliters of the PCR reaction were added to a cloning kit ligation reaction (The Ligator, R&D Systems, Abingdon, UK), and bacterial clones were raised according to the manufacture’s instructions. For the V4-V5 domains, the sense primer was V45f (5′-ATAGTGCTTCCTGCTGCTCCCAA-3′), antisense V45r (5′-GGCTAACTAGGGA ACCCACTG-3′) antisense primer was V45f (5′-CCTCAGGGAGGCACCCAGAAATTG-3′) and antisense primer was V45r (5′-ATAGTGCTTCCTGCTGCTCCCAA-3′). Amplification reactions were carried out for 40 PCR cycles. Sequencing was performed by fluorescence-labeled dNTPs in an automated sequencer (Model 373A, Perkin Elmer Cetus, Norwalk, CT). The sequences have been deposited with GenBank under Accession Nos. AF333189–AF333232.

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REFERENCES

Alfano, M., Schmidtmaierova, H., Amella, C. A., Pushkarsky, T., and


